

ARTICLE



Sequential interspecies interactions affect production of antimicrobial secondary metabolites in *Pseudomonas protegens* DTU9.1

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Soil and rhizosphere microbiomes play important roles in suppression of plant pathogens through production of antagonistic secondary metabolites, yet mechanisms that determine the strength of pathogen control are not well understood. Many *Pseudomonas* species are associated with soil and rhizosphere microbiomes, and their ability to suppress pathogens is well documented. Here, we investigate how interactions within the *Pseudomonas* genus affect their production of antimicrobial metabolites. From a biosensor-based screen, we identify *P. capeferrum* species as capable of modulating secondary metabolite production in *P. protegens*. We show that *P. capeferrum* alters production of pyoluteorin and 2,4-diacetylphloroglucinol (DAPG) in *P. protegens* via two distinct and sequential mechanisms that depends on spatial proximity of the two species. Specifically, *P. capeferrum* secretes a diffusible signal that induce pyoluteorin production up to 100-fold in neighboring *P. protegens* colonies. In contrast, the interaction results in reduced DAPG production, but only within mixed-species colonies. Additionally, we found that increased pyoluteorin production and cell lysis of *P. capeferrum* is required for inhibition of DAPG production, suggesting that pyoluteorin-facilitated antibiosis of *P. protegens* on *P. capeferrum* leads to release of cell-associated metabolites and subsequent inhibition of DAPG production in *P. protegens*. As the interaction modulates in vitro bioactivity of the species, genus-specific interactions may assist in improving efficacy of biocontrol strains and consortia.

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INTRODUCTION

Soil- and rhizosphere-associated microbiomes play critical roles in plant development and health, for example by production of antimicrobial metabolites that suppress plant diseases by antagonizing growth of phytopathogens [1–3]. Understanding the processes that either potentiate or reduce this antagonism is essential for predicting the role and function of bacterial communities in controlling plant–pathogen interactions, and for enabling biological control applications in agriculture. Several studies have shown that antagonistic activity towards plant pathogens is related to bacterial diversity in ways that are not well understood [4–8]. For example, using defined consortia of *Pseudomonas* species it has been shown that increasing genotypic richness (i.e. increasing number of genotypically similar consortia members) correlate with increased disease suppressiveness and antagonistic activity towards phytopathogens [5, 7]. In contrast, another study has demonstrated that an increasing richness of distantly related *Pseudomonas* spp. is correlated with reduced pathogen inhibition [8]. These and other examples suggest that genus-specific interactions among *Pseudomonas* species can affect the strengths of their antagonistic activities and highlights the importance in understanding the underlying microbial interaction processes.

Pseudomonas spp. are a group of soil bacteria that play key roles in plant growth promotion and control of crop pathogens due to production of various antimicrobial metabolites [2, 4]. Among the

fluorescent pseudomonads, *Pseudomonas protegens* has been studied intensively due to its potential as biocontrol agent and its array of antimicrobial secondary metabolites, including 2,4-diacetylphloroglucinol (DAPG), pyoluteorin, pyrrolnitrin, orfamide A and pyoverdine [9, 10]. This bacterium has been shown to exhibit antimicrobial activity against pathogenic bacteria, fungi and oomycetes [11–13]. At the mechanistic level, the biosynthesis and regulation of these metabolites has been well characterized in *P. protegens*, including that of DAPG and pyoluteorin. For the biosynthesis of DAPG, three molecules of malonyl-CoA are initially converted to phloroglucinol (PG) by the polyketide synthase, PhlD. PG is subsequently acetylated by the PhlACB enzyme complex once to mono-acetylphloroglucinol (MAPG) and twice to DAPG [14, 15]. The biosynthetic operon, *phlACBDE*, is under transcriptional control by the PhlF repressor. Additionally, Bottiglieri et al. found that a gene located upstream, *phlG*, encodes the PhlG enzyme, which degrades intracellular DAPG to MAPG [16]. The transcription of *phlG* is under control of the PhlH repressor [17]. The biosynthesis of pyoluteorin starts with L-proline, which is converted to a dichloro-pyrrole by a three-step enzymatic process by PltF, PltE and PltA. Pyoluteorin is formed by the addition of a resorcinol ring to the dichloro-pyrrole moiety catalyzed by the enzyme complex, PltBCG [18, 19]. Transcription of the biosynthetic operon, *pltABCDEF*, is activated by PltR. Recently, Yan et al. reported that the biosynthesis of DAPG and pyoluteorin is connected in *P. protegens*

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Pf-5, where the halogenase, PltM, converts the precursor, PG, from the DAPG pathway to PG-Cl and PG-Cl₂ [10]. These molecules bind to the PltR activator, which in turn activates transcription of the pyoluteorin biosynthetic gene cluster.

The regulation of DAPG and pyoluteorin biosynthesis has been studied extensively in strains of *P. protegens*. It was demonstrated that these antimicrobial metabolites function in intra- and intercellular signaling circuits, as both molecules induced their own biosynthesis [20–22]. Auto-induction of DAPG was demonstrated in situ on wheat roots, as expression from the *PphIA* promoter was induced in two distinct *Pseudomonas* spp. by co-inoculation with a DAPG-producing strain [22]. Similarly, Brodhagen et al. reported that pyoluteorin functioned in positive autoregulation in *P. protegens* Pf-5 by inducing transcription of its respective biosynthetic gene cluster during growth on cucumber seedlings [21]. Recently, it was further reported that the intermediate PG in the biosynthesis of DAPG contributes to the regulation of production of both DAPG and pyoluteorin in a concentration-dependent manner [23, 24]. Additionally, production of both metabolites requires activation from the global regulatory system, Gac/Rsm [25]. Overall, previous work in the field has illuminated the complex regulatory intra- and intercellular mechanisms involved in fine-tuning the production of DAPG and pyoluteorin in strains of *P. protegens*. Additionally, Dubuis et al. demonstrated that interspecies interactions between distantly related *Pseudomonas* spp. stimulated antimicrobial activity of *P. protegens* CHA0 against *Bacillus subtilis* presumably via Gac/Rsm-induced production of DAPG [26]. However, the effects of microbial interactions and their underlying molecular mechanisms affecting the biosynthesis of DAPG and pyoluteorin has remained less characterized.

In this study we investigate the effects of interspecies interactions between the soil isolate, *P. capeferrum* F8, and a potential biocontrol agent, *P. protegens* DTU9.1, on the production of the two antimicrobial metabolites, DAPG and pyoluteorin. Our results show that cocultivation of the two bacteria in a biofilm on agar surfaces inhibits the production of DAPG, while significantly inducing the biosynthesis of pyoluteorin in *P. protegens*. We reveal that the two regulatory mechanisms occur from two independent signals – a cell-associated signal and an extracellular metabolite from *P. capeferrum* F8. Moreover, we demonstrate that the cocultivation of the two *Pseudomonas* species enhances the antimicrobial efficiency against two known bacterial phytopathogens. Lastly, we show that the antibiosis of *P. protegens* DTU9.1 on *P. capeferrum* F8 due to pyoluteorin production leads to cell lysis of F8 and release of an unknown metabolite inhibiting DAPG production. Overall, our study shows that bacterial secondary metabolites can be part of intricate interaction networks (even at dual species level) that may result in a multitude of changes including repression and induction of biosynthetic gene clusters. The dynamic, sequential interactions and resulting changes in chemical output (i.e. levels of secondary metabolites) from these interactions may help to further advance our understanding of e.g. microbial turnover in microbiomes and activities of biocontrol strains.

RESULTS

P. protegens alters its secondary metabolism in coculture with a *P. capeferrum* soil isolate

In a previous screen for DAPG-producing *Pseudomonas* species from grassland soil samples, we identified a small fraction of isolates (9/864) unable to produce DAPG themselves, due to lack of the *phlACBDE* biosynthetic gene cluster (verified by PCR targeting *phlD* and chemical analysis). Yet the nine isolates were capable of eliciting a response in an engineered DAPG-responsive, PhlF-based whole-cell biosensor [27]. We hypothesized that these isolates had the potential to modulate DAPG production in *Pseudomonas* species known to produce DAPG (such as *P. protegens*) and to affect secondary metabolite production through unknown cell-cell

interactions. Preliminary cocultivation experiments between the nine isolates and a DAPG producer, *P. protegens* DTU9.1, suggested that two isolates could inhibit DAPG production below the limit of detection. Based on their *rpoD* sequences, these two isolates were identified as *P. capeferrum* spp. and we selected one of them, *P. capeferrum* F8, for further studies of its ability to modulate secondary metabolite production in *P. protegens* DTU9.1.

To investigate further, *P. capeferrum* F8 and *P. protegens* DTU9.1 were cocultivated in a mixed-species colony on a KBmalt agar surface. Quantitative analysis of DAPG and pyoluteorin was carried out using liquid chromatography-high resolution mass spectrometry (LC-HRMS) on agar plugs covering the entire microbial colony. The concentrations of DAPG and pyoluteorin were normalized to colony forming units (CFU) of the producing bacterium (Fig. 1A). Production of DAPG was inhibited below the limit of detection (LOD) in coculture with *P. capeferrum* F8 (Fig. 1A). Contrarily, the soil isolate significantly induced a 10–100 fold increase in pyoluteorin production in *P. protegens* DTU9.1 after 24 h ($p = 4.8 \times 10^{-2}$) and 48 h ($p = 7.8 \times 10^{-3}$). To examine the coculture dynamics, *P. capeferrum* F8 and *P. protegens* DTU9.1 were tagged with the Tn7 transposon from pBG42-*mKate* and pBG42-*gfp*, respectively. After 24 h of growth, *P. capeferrum* F8 constituted most of the bacterial colony ($p = 1.1 \times 10^{-2}$). On the second day of incubation, the ratio of the two bacteria had shifted towards an even distribution, as *P. protegens* DTU9.1 increased in abundance, while the abundance of *P. capeferrum* F8 decreased. However, despite this shift in coculture dynamics, DAPG production remained below the detection limit (Fig. 1A).

P. capeferrum F8 affects metabolite production in *P. protegens* with two independent signals

Based on the coculture interaction results above, we attempted to attribute the altered secondary metabolism in *P. protegens* DTU9.1 to secretion of a metabolite or signal from *P. capeferrum* using a distance assay on KBmalt agar. First, *P. capeferrum* F8 was grown for 48 h in bacterial colonies, followed by inoculating *P. protegens* DTU9.1 at a distance of 5, 12.5 and 20 mm from the edge of the F8 colony. Agar plugs covering the colony of *P. protegens* DTU9.1 were extracted after an additional 24 h of incubation. The concentrations of DAPG produced by *P. protegens* DTU9.1 was unaffected by diffusible signals from *P. capeferrum* F8, whereas pyoluteorin production was induced in a distance-dependent manner (Fig. 2A). *P. protegens* DTU9.1 was grown under axenic conditions as a control. To fit an overall non-linear regression model to the data, the control was assigned a 100 mm distance equivalent of the diameter of an entire petri dish. The induction of pyoluteorin as a function of distance is well described by a one phase decay function ($R^2 = 0.997$) having a decay rate of -0.216 ($p = 8.1 \times 10^{-5}$). Closer inspection of the individual data points revealed that pyoluteorin production in the two closest colonies (5 and 12.5 mm) was induced significantly compared to the control as evidenced by ANOVA followed by Dunnett's test ($p = 2.1 \times 10^{-5}$ and $p = 3.2 \times 10^{-3}$, respectively), whilst the 20 mm colony was not significantly induced.

The secreted signal from *P. capeferrum* F8 was shown to induce the transcription of the pyoluteorin biosynthetic gene cluster from the *PphIL* promoter, and this effect decreases with increasing distance between the bacterial colonies (Fig. 2C). Additionally, the relative fluorescence of *P. protegens* DTU9.1 harboring pSE-VA::PphIL-*gfp* was measured with ImageJ after 24 h of cultivation (Fig. 2D). This showed that promoter activity was significantly induced at the 5 and 12.5 mm spots ($p = 4.2 \times 10^{-9}$ and $p = 4.4 \times 10^{-4}$, respectively), while the transcriptional activity was not significantly different from the control at the 20 mm spot. Overall, these findings suggests that DAPG inhibition and induction of pyoluteorin biosynthesis are affected by two independent molecular mechanisms. The signal inducing pyoluteorin biosynthesis is secreted by *P. capeferrum* F8, whereas

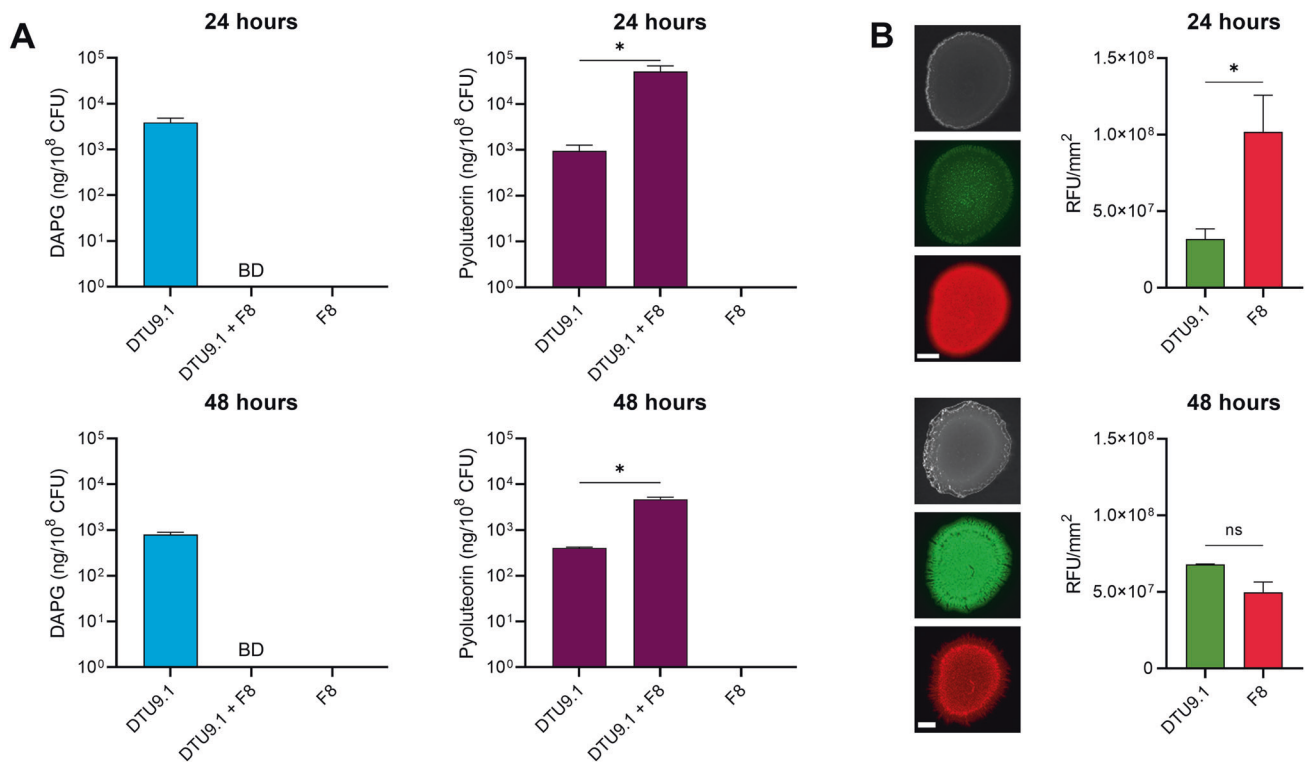


Fig. 1 *P. protegens* alters its secondary metabolism in coculture with *P. caeperrum* F8 on solid surface. **A** Concentration of DAPG and pyoluteorin produced by *P. protegens* DTU9.1 after 24 and 48 h of growth on KBmalt agar. BD = Below detection (Student's *t*-test: **p* < 0.05). **B** Coculture dynamics of *P. caeperrum* F8 (red – *mKate*) and *P. protegens* DTU9.1 (green – *gfp*) on KBmalt. Top: Bright field image. Middle: *gfp* channel. Bottom *mKate* channel. Scale bar corresponds to 2 mm. Pictures are representative of three biological replicates with similar results. Fluorescence images were obtained with 500 ms exposure time. Relative fluorescence per colony for both channels was estimated with ImageJ.

direct cell-to-cell interaction may be required for the inhibition of DAPG as achieved in a mixed species colony.

Distance-dependent induction of pyoluteorin biosynthesis is observed among closely and distantly related bacteria

To explore the breadth of distance-dependent signaling from related *Pseudomonas* species, as well as unrelated bacteria, we tested six additional isolates of *Pseudomonas* and five phylogenetically distant bacteria for their ability to induce pyoluteorin biosynthesis in *P. protegens* DTU9.1. For the six *Pseudomonas* strains, *P. putida* F1 was included as a close relative to *P. caeperrum* F8, as part of the *P. putida* group. Moreover, we included five strains (*P. helmanticensis* DTU12.3, *P. jessenii* DTU3.1, *P. kilonensis* 5.21, *P. protegens* DTU9.1 and *P. proteolytica* DTU12.1) representing diverse soil *Pseudomonas* species of the *P. fluorescens* group belonging to five separate subgroups (*P. koreensis*, *P. jessenii*, *P. corrugata*, *P. protegens* and *P. gessardii*). The phylogenetic association between the *Pseudomonas* isolates in relation to known type strains [28] was evaluated based on part of the *rpoD* house-keeping gene (Figure S1). Four of the six tested *Pseudomonas* isolates induced a significant activation of the *PpLT* promoter in a distance-dependent manner (Fig. 3). However, two of the isolates (*P. kilonensis* 5.21 and *P. protegens* DTU9.1) did not induce promoter activity in *P. protegens* DTU9.1 harboring pSEVA237::PpLT-*gfp*. Additionally, we investigated if species outside the *Pseudomonas* genus could induce pyoluteorin biosynthesis in a distance-dependent manner. To this end, we tested a rapidly growing *E. coli* strain, as well as four commonly isolated soil bacteria, including *Pectobacterium* sp. D749, *Rhodococcus globerulus* D757, *Stenotrophomonas indicatrix* D763 and *Chryseobacterium* sp. D764 [29]. Both *Pedobacter* sp. D749 and *S. indicatrix* D763 secreted a signal capable of inducing pyoluteorin

biosynthesis in *P. protegens* DTU9.1. These findings could indicate that *P. protegens* responds to a more general signal or cue from closely, as well as distantly related bacteria, by upregulating the production of pyoluteorin.

DAPG and pyoluteorin biosynthesis are affected by two independent signals from *P. caeperrum* F8

The results described above suggested the presence of two independent signals; a secreted signal inducing pyoluteorin biosynthesis and a signal requiring cell-to-cell contact between the two bacteria resulting in inhibition of DAPG production in *P. protegens* DTU9.1. Thus, we hypothesized that a supernatant of an outgrown *P. caeperrum* F8 culture would have the ability to induce pyoluteorin production in *P. protegens* DTU9.1, whereas molecules extracted from a lysate of the corresponding F8 cell-pellet would inhibit DAPG production. Exposing *P. protegens* DTU9.1 to 50% v/v fresh KBmalt broth and supernatant of a 48 h culture of *P. caeperrum* F8 significantly induced pyoluteorin production, while DAPG biosynthesis was unaffected (Fig. 4A). Moreover, the inducing molecule appeared to be heat sensitive and completely lost its activity following 15 min of autoclaving at 121 °C (Fig. 4B). However, treatment with proteinase K (30 U/ml) did not significantly reduce the activity of the supernatant, suggesting that the inducing molecule is not of proteinaceous nature. In contrast, exposing *P. protegens* DTU9.1 to 50% v/v fresh KBmalt broth and sterile water did not induce transcription from the *PpLT* promoter, which clearly indicated that the supernatant from *P. caeperrum* F8 contained a signal capable of inducing pyoluteorin production in *P. protegens*.

In order to test if cell-associated molecules could affect the secondary metabolism of *P. protegens* DTU9.1, we exposed *P. protegens* to molecules extracted from the lysate of a *P. caeperrum*

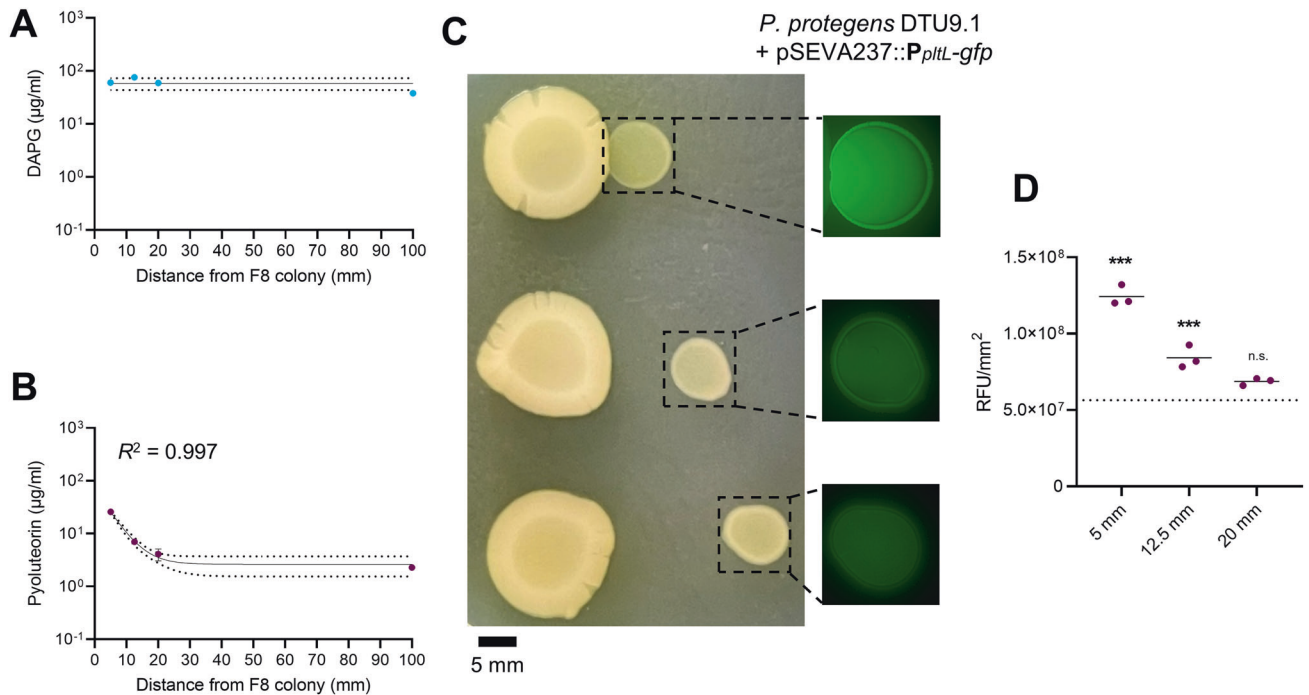


Fig. 2 *P. capeferrum* F8 induces pyoluteorin biosynthesis in a distance-dependent manner. Concentration of DAPG (**A**) and pyoluteorin (**B**) was measured in two independent experiments measured with HR-LCMS. The control represents *P. protegens* DTU9.1 grown in isolation on KBmalt agar (set to 100 mm distance to fit the model). Dotted lines represent the 95% confidence intervals of the mathematical model. **C** Distance assay with *P. protegens* DTU9.1 harboring pSEVA237::PpLT-gfp indicating that increased pyoluteorin production was due to induced transcription of the pyoluteorin biosynthetic gene cluster in a distance-dependent manner. Pictures are representative of three biological replicates with similar results. Fluorescence images were obtained with 500 ms exposure time. **D** Relative fluorescence per colony was estimated with ImageJ. The dotted line represents the average fluorescence measured in the control cultivated axenically (Dunnett's test compared to control: *** $p < 0.001$).

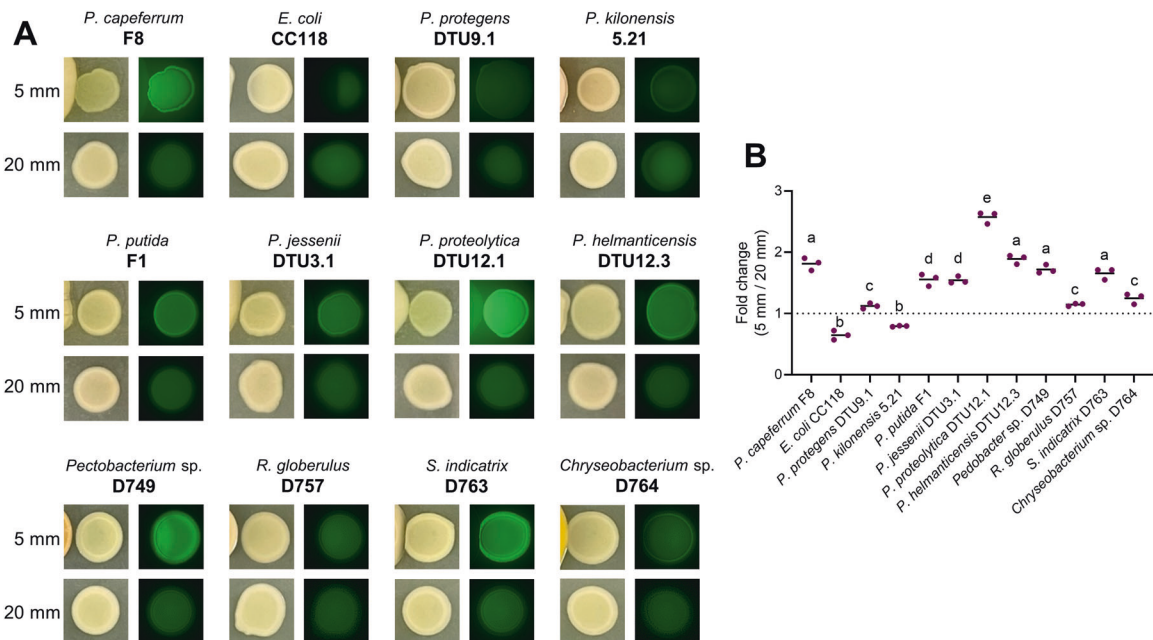


Fig. 3 Distantly related species induce pyoluteorin biosynthesis in *P. protegens* DTU9.1. **A** Distance assay with *P. protegens* DTU9.1 harboring pSEVA237::PpLT-gfp. Pictures are representative of three biological replicates with similar results. Fluorescence images were obtained with 500 ms exposure time. **B** Fold change in relative fluorescence of *P. protegens* DTU9.1 harboring pSEVA237::PpLT-gfp cultivated 5 and 20 mm from the interacting bacteria. Relative fluorescence per colony was estimated with ImageJ from three biological replicates. The dotted line represents the control of *P. protegens* DTU9.1 cultivated alone. Letters above columns indicate treatments significantly different from one another, as determined by ANOVA and subsequent Tukey *post-hoc* analysis ($p < 0.05$).

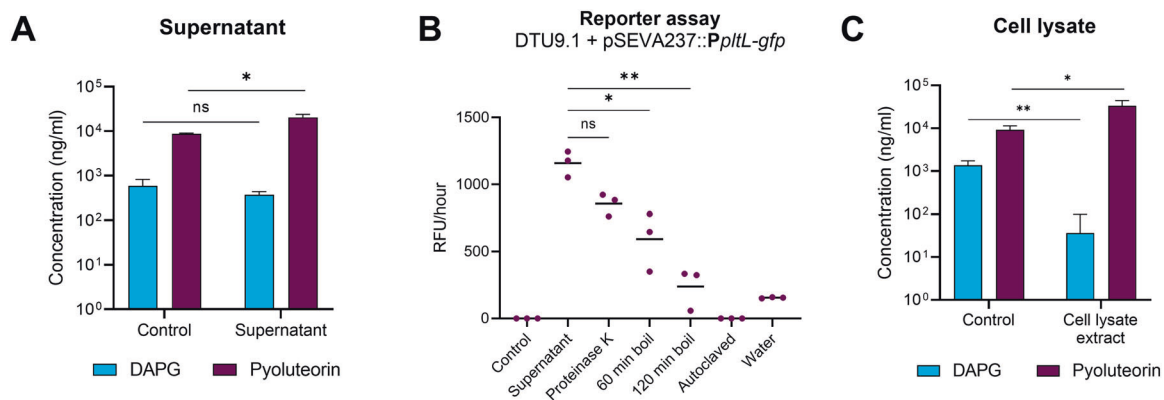


Fig. 4 DAPG and pyoluteorin biosynthesis are affected by two independent signals from *P. caeperrum* F8. **A** Concentration of DAPG and pyoluteorin produced by *P. protegens* DTU9.1 cultured in KBmalt (control) or 50% v/v sterile-filtered supernatant from a 48 h culture of *P. caeperrum* F8. **B** Supernatant from a 48 h culture of *P. caeperrum* F8 was treated with proteinase K, boiled for 60 or 120 min at 100 °C or autoclaved for 15 min at 121 °C. *P. protegens* DTU9.1 harboring pSEVA237::PpLT-gfp was exposed to the treated supernatants, sterile water or fresh KBmalt media as control. Promoter activity was measured as the increase in relative fluorescence per h during stationary phase. **C** Concentration of DAPG and pyoluteorin produced by *P. protegens* DTU9.1 cultured in KBmalt supplemented with methanol as control or crude ethyl acetate extracted cell lysate of *P. caeperrum* F8. Significance levels were calculated with the Student's *t*-test (* $p < 0.05$, ** $p < 0.005$).

F8 cell-pellet. To this end, *P. protegens* DTU9.1 was cultivated in three independent biological replicates in KBmalt broth supplemented with methanol as control or crude lysate extract. The supernatants were collected after 24 h and analyzed for the presence of DAPG and pyoluteorin with LC-HRMS (Fig. 4C). The crude cell lysate extract significantly inhibited DAPG production 38-fold ($p = 3.3 \times 10^{-3}$), where the DAPG concentration in two out of the three replicates was below the LOD. The production of pyoluteorin was induced 4-fold ($p = 2.2 \times 10^{-2}$). This suggests that cell lysis of *P. caeperrum* F8 leads to release of cell-associated metabolites inhibiting the production of DAPG. The minor induction of pyoluteorin biosynthesis is most likely due to small amounts of extracellular signaling molecules associated with *P. caeperrum* F8 cells during washing pre-lysis or that the inducing molecule is present intracellularly in low concentrations.

The antibiosis of *P. Protegens* on *P. caeperrum* F8 leads to inhibition of DAPG production

Based on these results, we hypothesized that antibiosis of *P. protegens* DTU9.1 on *P. caeperrum* F8 in mixed-species cocultures could facilitate the release of cell-associated metabolites from *P. caeperrum* leading to inhibition of DAPG production. We further hypothesized that the antimicrobial metabolite, pyoluteorin, was responsible for the antibiosis. Initially, we evaluated the minimal inhibitory concentration (MIC) of DAPG and pyoluteorin towards *P. caeperrum* F8 (Fig. S2). This revealed that pyoluteorin was toxic towards *P. caeperrum* F8, inhibiting growth at 64 µg/ml, whereas the MIC of DAPG exceeded the tested concentrations (> 128 µg/ml). To test the hypothesis that pyoluteorin caused antibiosis of *P. caeperrum* F8, we cultivated *P. protegens* DTU9.1 WT and a Δ pLT mutant in mono- and coculture colonies with *P. caeperrum* F8 on a KBmalt agar surface. The Δ pLT deletion mutant of *P. protegens* DTU9.1 was incapable of converting phloroglucinol (PG) to PG-Cl and PG-Cl₂ [10], which in turn rendered the bacterium unable to initiate transcription of the pyoluteorin biosynthetic gene cluster. Cocultivation of *P. caeperrum* F8 and the Δ pLT deletion mutant of *P. protegens* DTU9.1 restored production of DAPG to levels comparable to or higher than monocultures of *P. protegens* DTU9.1 WT and Δ pLT mutant (Fig. 5A, D), demonstrating that pyoluteorin production is required for the observed suppression of DAPG production.

Cell counts of the bacteria residing in the mixed colonies further revealed that the absence of pyoluteorin in the Δ pLT mutant improved the ability of the two *Pseudomonas* species to co-exist

(Fig. 5B, E). The abundance of *P. caeperrum* F8 was significantly higher in coculture with the Δ pLT mutant compared to wildtype *P. protegens* DTU9.1 after 24 h ($p = 2.8 \times 10^{-2}$) and 48 h ($p = 2.5 \times 10^{-2}$). Furthermore, the concentration of pyoluteorin in the coculture between the two bacteria reached levels comparable to the MIC (Fig. 5A), which supports the model that pyoluteorin causes antibiosis of *P. caeperrum* F8. This data indicates that the cell-associated, DAPG-inhibiting signal from *P. caeperrum* F8 is located intracellularly requiring cell lysis for its release. Additionally, the absence of pyoluteorin production in the Δ pLT mutant did not affect the ability of *P. protegens* DTU9.1 to proliferate in the mixed species coculture (Fig. 5B, E). Lastly, restoration of DAPG production in the cocultivation of *P. caeperrum* F8 and the Δ pLT deletion mutant of *P. protegens* DTU9.1 was visualized utilizing the whole-cell DAPG biosensor harboring pSEVA225::DAPG_{lacZ} (Fig. 5C, F). Monocultures of *P. protegens* DTU9.1 WT and Δ pLT mutant yielded a strong response as evidenced by a clear blue halo surrounding the colony of *Pseudomonas*. Cocultivation of *P. caeperrum* F8 and the Δ pLT deletion mutant generated a similar response after 48 h of incubation but only a slight induction was observed after 24 h. This suggests that the biosensor response had an unexpected delay, since the LC-HRMS data showed restoration of DAPG production after 24 h (Fig. 5A). Taken together, these findings suggest that pyoluteorin facilitates lysis of *P. caeperrum* F8 leading to the release of one or more metabolites capable of inhibiting DAPG production in *P. protegens* DTU9.1.

The antimicrobial activity of *P. protegens* DTU9.1 against bacterial phytopathogens is increased in coculture with *P. caeperrum* F8

Previous research showed that using a diverse consortium of *Pseudomonas* species as biocontrol inoculum can increase disease suppressiveness [7]. Given the changes to secondary metabolite production in cocultures between *P. protegens* DTU9.1 and *P. caeperrum* F8, it could suggest that such cocultures would exhibit altered suppression of growth of DAPG and pyoluteorin sensitive microorganisms compared to monoculture. To test this, *Pseudomonas* species were cultivated in mono- and coculture colonies on KBmalt agar for 48 h to allow for metabolite production prior to chloroform-mediated cell inactivation to avoid undesirable swarming. Subsequently, the plates were covered with a soft agar layer inoculated with two known phytopathogenic bacteria, *Pectobacterium carotovorum* and *Dickeya solani*. Inhibition was

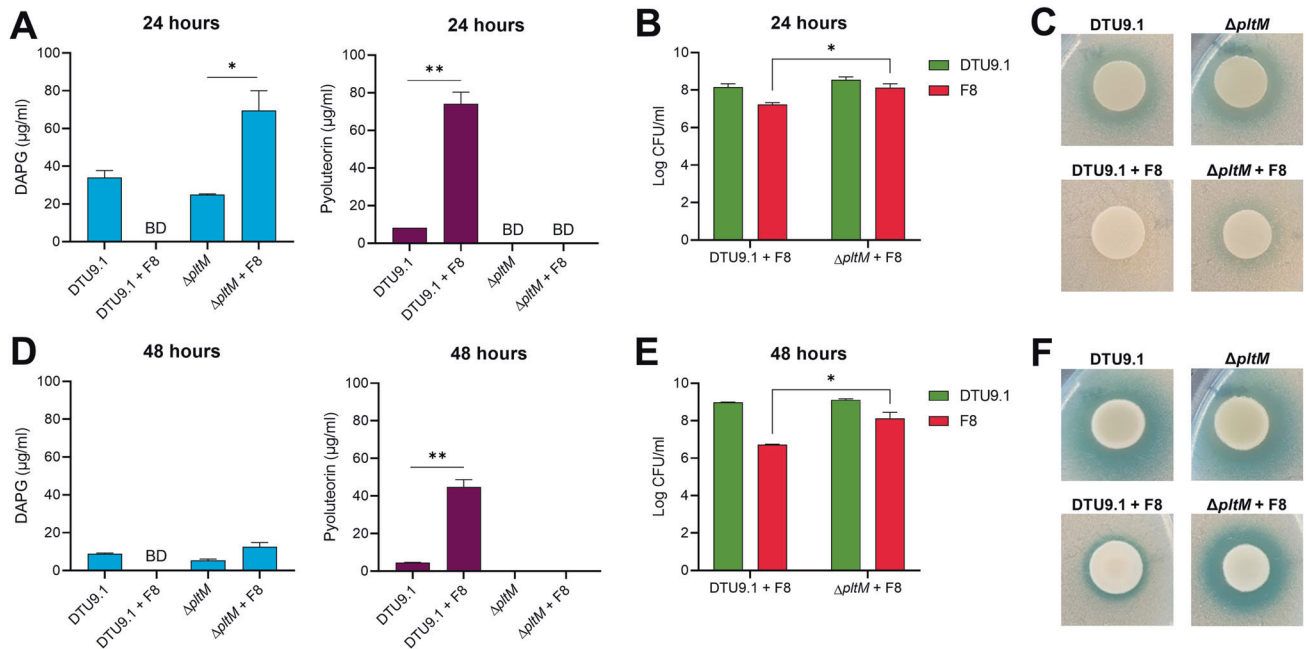


Fig. 5 Deletion of *pltM* restores DAPG production in coculture between *P. protegens* DTU9.1 and *P. caeperrum* F8. **A, D** Concentration of DAPG and pyoluteorin produced by *P. protegens* DTU9.1 and the $\Delta pltM$ mutant in mono- and coculture with *P. caeperrum* F8 after 24 h (**A**) and 48 h (**D**). BD = Below detection limit **B, E** Abundance of *P. protegens* DTU9.1 or $\Delta pltM$ mutant and *P. caeperrum* F8 in mixed colonies on KBmalt agar after 24 h (**B**) and 48 h (**E**). Values represent means of two independent experiments. **C, F** *P. protegens* DTU9.1 and $\Delta pltM$ mutant in mono- and coculture with *P. caeperrum* F8 in bacterial colonies on top of a lawn of the biosensor harboring pSEVA225::DAPG_{lacZ} on KBmalt + Xgal65 agar after 24 h (**C**) and 48 h (**F**). Significance levels were calculated with the Student's *t*-test (**p* < 0.05, ***p* < 0.005).

evaluated based on the size and turbidity of the clearing zone arising after an additional 24 h of incubation (Fig. 6A). Cocultivation leads to a significant increase in antimicrobial effectiveness against both plant pathogens compared to both monocultures of *Pseudomonas* ($p = 1.1 \times 10^{-3}$ and $p = 3.4 \times 10^{-3}$ for *P. carotovorum* and *D. solani*, respectively) (Fig. 6B). To examine if the induced production of pyoluteorin was the cause of increased bioactivity, the *pltM* gene was knocked out in *P. protegens* DTU9.1 resulting in absence of pyoluteorin biosynthesis [10]. Bioactivity against both plant pathogens was lost in the $\Delta pltM$ mutant in monoculture and greatly reduced in coculture with *P. caeperrum* F8 compared to wildtype *P. protegens* DTU9.1 (Fig. 6).

DISCUSSION

The biosynthesis of secondary metabolites has been extensively studied in strains of *P. protegens*, which has led to the characterization of complex intra- and intercellular regulatory mechanisms governing the fine-tuned production of specific metabolites [20–22, 24]. However, bacteria most often reside in microbial communities in which communication mediated through chemical signaling adds another layer of regulatory complexity. Additionally, previous studies have demonstrated conflicting results correlating *Pseudomonas* spp. richness and the effects on ecologically relevant mechanisms mediated by secondary metabolites (e.g. antimicrobial activity and plant protection) [5–8]. This supports the potential for uncharacterized, genus-specific interactions among *Pseudomonas* spp. affecting their secondary metabolism. Here we report on the effects of interspecies interactions between two fluorescent *Pseudomonas* on the production of DAPG and pyoluteorin in *P. protegens* DTU9.1. Our work sheds light on interspecies interactions between soil *Pseudomonas* that affect the biosynthesis of antimicrobial secondary metabolites. This further emphasizes the necessity of studying microbial interactions to broaden our current understanding of the natural role and biosynthesis of secondary

metabolites, while bridging the anticipated in vitro and actual in situ efficacy of biocontrol agents.

In this study, the cocultivation of *P. caeperrum* F8 and a potential biocontrol agent, *P. protegens* DTU9.1, led to induced expression of the pyoluteorin biosynthetic gene cluster, while production of DAPG was inhibited (below LOD). We propose the sequential signaling model (Fig. 7) in which *P. protegens* initially responds to an extracellular, diffusible signal from *P. caeperrum* by inducing the biosynthesis of the antibacterial metabolite, pyoluteorin. The increased local concentration of pyoluteorin exceeds tolerable levels for *P. caeperrum*, leading to cell disruption. As a result of lysis within the *P. caeperrum* population, an intracellular signal (from *P. caeperrum*) is released, which subsequently inhibits production of DAPG in *P. protegens* DTU9.1. We speculate that the inhibition of DAPG production could be related to a yet uncharacterized ecological function, as the absence of DAPG has no apparent benefit to either organism in this dual species interaction. Previously, it was reported that *P. aeruginosa* releases an intracellular signal upon cell lysis, which in turn induces the biosynthesis of antibacterial metabolites in neighboring kin cells [30]. In the present study, however, we describe a dynamic, sequential interaction between two unrelated species of *Pseudomonas* affecting the biosynthesis of multiple secondary metabolites.

Bacterial communication mediated via chemical signaling is a concept that has been extensively studied. For instance, previous research has shown that dual-species interactions between culturable soil bacteria commonly result in increased antimicrobial activity, due to induced antibiotic production, compared to monocultures [31, 32]. An explanation for the distance-dependent induction of pyoluteorin observed in this study could be related to an interaction arising from bacterial competition sensing [33–35]. Such interactions often involve secretion of toxic metabolites proposed to have hormetic properties (e.g. stimulatory at subinhibitory concentrations and antagonistic at intolerable concentrations) [36]. At subinhibitory concentrations the

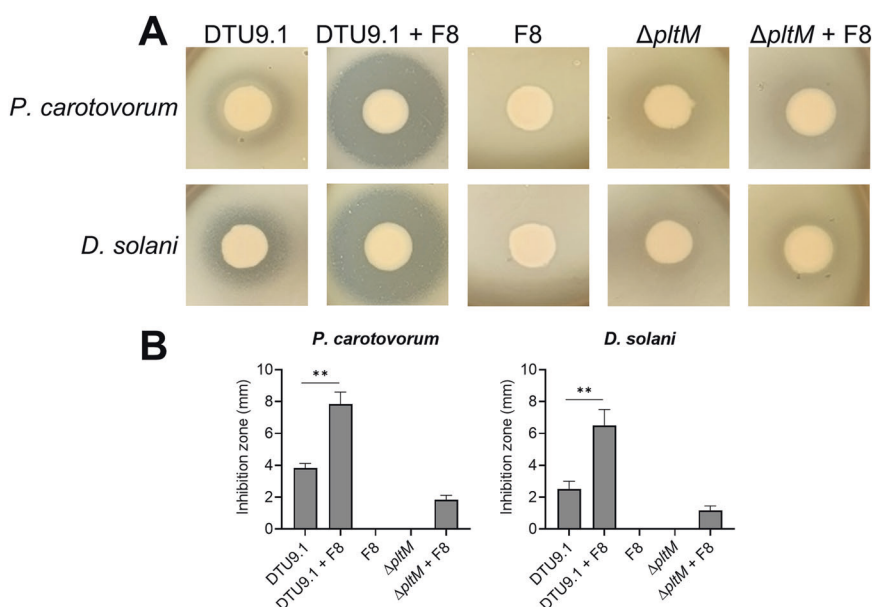


Fig. 6 The antimicrobial effectiveness of *P. protegens* DTU9.1 against bacterial phytopathogens increases in coculture with *P. caepiferrum* F8. **A** The antimicrobial activity of *P. protegens* DTU9.1 towards two bacterial phytopathogens, *P. carotovorum* and *D. solani*, was enhanced in coculture with *P. caepiferrum* F8. The $\Delta pltM$ mutant of *P. protegens* DTU9.1 was unable to produce pyoluteorin. Pictures are representative of three biological replicates with similar results. **B** Zones of inhibition were measured for three biological replicates (Student's *t*-test: ***p* < 0.005).

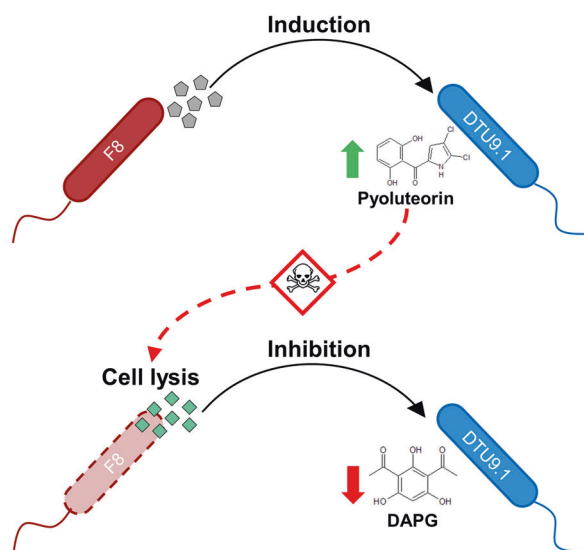


Fig. 7 Schematic overview of the altered secondary metabolism in *P. protegens* DTU9.1 in response to cocultivation with *P. caepiferrum* F8. *P. caepiferrum* F8 secretes a metabolite (grey pentagons), which induces biosynthesis of pyoluteorin in *P. protegens* DTU9.1. The increased concentration of pyoluteorin is toxic towards *P. caepiferrum* F8 leading to cell lysis and release of an intracellular metabolite (cyan diamonds), which in turn inhibits production of DAPG in *P. protegens*.

toxic metabolites may act as cues of nearby microbial danger, inducing responses in the sensing bacterium, such as modifications to its own secondary metabolome [35–38]. As demonstrated recently, competitive interactions are commonly observed in interspecies interactions among closely related species, likely owing to similar nutrient preferences [39]. Dubuis et al. showed a Gac/Rsm-mediated induction of antimicrobial activity in *P. protegens* CHA0 resulting from interspecies interactions [26]. In this study we have shown that *P. protegens* DTU9.1 induces

biosynthesis of pyoluteorin in response to a secreted signal from *P. caepiferrum* F8 (Fig. 2), as well as diverse species of *Pseudomonas* and at least two other species of commonly isolated soil bacteria (Fig. 3). On the other hand, lack of induction was observed for several species, including other *Pseudomonas* strains and distantly related bacteria. Moreover, we could demonstrate that exposing *P. protegens* DTU9.1 to the supernatant of a *P. caepiferrum* F8 culture induced biosynthesis of pyoluteorin (Fig. 4A). This supports the hypothesis that *P. protegens* DTU9.1 responds to a secreted signal rather than alterations in media composition caused by the interacting species. These findings suggest that *P. protegens* DTU9.1 responds to a more general bacterial cue, indicating the presence of a competing species. We could further show that the signal appeared heat sensitive, as boiling the supernatant significantly reduced the ability to induce transcription from the *PplT* promoter, while autoclaving completely removed the ability to induce (Fig. 4B). Contrarily, treatment of the supernatant with proteinase K did not significantly reduce the ability to induce pyoluteorin biosynthesis, indicating that the secreted signal from *P. caepiferrum* F8 is not of proteinaceous nature. Treatment of the supernatant with other enzymes, including α -amylase and lipase, could further aid in deducing the chemical nature of the secreted signal as recently demonstrated by Yang et al. [40]. In addition to enzymatic treatment, we also sequenced and *de novo* assembled the genome of *P. caepiferrum* F8 (see Methods) to identify candidate genes required for biosynthesis of potential secreted signals. This analysis revealed the presence of the *ppuL-rsaL-ppuR* quorum sensing (QS) system [41] and an unknown polyketide synthase. Previous research has shown that QS molecules display varying levels of promiscuity, as they interact with both intra- and interspecies receptors [42]. As described above, secreted secondary metabolites with antimicrobial properties, including polyketide-derived metabolites, have been associated with danger signals sensed by neighboring microorganisms [33, 34, 36]. However, knock-out mutants either with a deficient QS system or lacking the polyketide synthase did not affect the ability of *P. caepiferrum* F8 to induce pyoluteorin biosynthesis in *P. protegens* DTU9.1. Thus, further investigations are necessary to identify the secreted signal from *P. caepiferrum* F8 capable of inducing pyoluteorin production in *P. protegens*.

The biosynthesis of DAPG in *Pseudomonas* is under tight intra- and intercellular regulation and studies have shown that metabolites from interacting microbes can affect its production [20, 43]. Schnider-Keel et al. showed that salicylate, a molecule with structural similarities to DAPG, could also inhibit biosynthesis of DAPG [20]. Interestingly, Li et al. found that *P. putida* RW10S1 produced promysalin, which is a salicylate-containing antibiotic selectively targeting other *Pseudomonas* species [44]. Since species of *P. putida* are closely related to *P. capeferrum* [28], we speculated if our soil isolate, *P. capeferrum* F8, possessed the genes required for salicylate and promysalin production. However, a tBLASTn analysis revealed the absence of this biosynthetic gene cluster. The signal inhibiting DAPG production in *P. protegens* DTU9.1 could be either intracellular or membrane-associated in *P. capeferrum* F8 (Fig. 4C). However, the abundance of the signaling bacterium (*P. capeferrum* F8) was stably maintained at approximately 10^8 CFU/ml in the coculture with the Δ *pltM* mutant compared to coculture with WT *P. protegens* DTU9.1, where the abundance of F8 declined 10–30 fold over the course of 48 h (Fig. 5B, E). This could indicate that the inhibiting signal is located intracellularly, requiring cell lysis to be released. Nevertheless, biosynthesis of DAPG was restored when the Δ *pltM* mutant was cultured with *P. capeferrum* F8. This indicates that cell lysis of *P. capeferrum* F8 caused by the elevated pyoluteorin concentration in coculture with WT *P. protegens* DTU9.1 is necessary for the release of an intracellular signal and inhibition of DAPG production. Further analyses are needed to identify the intracellular chemical signal from *P. capeferrum* F8 capable of inhibiting DAPG production. Additionally, we observed significantly higher levels of DAPG after 24 h in the coculture between *P. capeferrum* F8 and the Δ *pltM* mutant compared to monoculture of the mutant (Fig. 5A). This could indicate that *P. capeferrum* F8 additionally has the ability to induce DAPG production. This could further aid in explaining our initial observation utilizing the DAPG-responsive, PhIF-based whole-cell biosensor. Thus, the biosensor could have been responding to the presence of this inducing molecule, however, during cocultivation of *P. protegens* DTU9.1 and *P. capeferrum* F8 the release of the intracellular and inhibiting molecule overshadowed the effect of the inducing molecule, ultimately resulting in inhibition of DAPG production. Overall, our work and previous studies underline the importance of investigating microbial interactions to further elucidate the complex roles of secondary metabolites in natural communities.

In the recent decades, employing specific species of isolated bacteria as microbial biocontrol agents has gained increased attention for the suppression of phytopathogenic fungi and bacteria as a sustainable alternative to potentially harmful pesticides. The effectiveness of such biocontrol inoculants relies in part on antimicrobial activity against plant pathogens, which can typically be attributed to the production of antimicrobial secondary metabolites. However, despite the improvements in *in vitro* screening for novel candidate biocontrol microbes, less than 1% end up as commercial products [45]. The lack of efficacy *in situ* may in part be due to yet uncharacterized interactions between members of the natural soil microbiome and the introduced biocontrol agent. The bacterium, *P. protegens*, has been studied for decades for its biocontrol properties [2], and we show here that chemical interactions with another *Pseudomonas* species commonly found in soil and rhizosphere microbiomes [46] can radically alter its production of antimicrobial secondary metabolites (Fig. 1) and its activity towards bacterial phytopathogens (Fig. 6). We speculate that such phenotype-altering chemical interactions play an important role in determining the efficiency of biocontrol. Indeed, there are several recent demonstrations of improved plant growth promoting properties, as well as the overall bioactivity against plant-pathogenic microorganisms, by use of mixed species inoculants. For example, Niu et al. demonstrated that a synthetic microbial community consisting of seven species greatly reduced

the severity of blight disease on maize seedling caused by the phytopathogen, *Fusarium verticillioides* [47]. Additionally, Zhuang et al. reported that a synthetic consortium consisting of six *Pseudomonas* spp. significantly affected plant growth promotion of radish seedlings [48]. Our results support that co-inoculation of two *Pseudomonas* strains; *P. protegens* DTU9.1 and *P. capeferrum* F8, augments the *in vitro* bioactivity against two known phytopathogens, *P. carotovorum* and *D. solani*, due to the increased production of pyoluteorin (Fig. 6). However, we also demonstrate a secondary microbial interaction leading to the inhibition of DAPG production. Natural conditions such as soil and the rhizosphere surrounding plant roots may facilitate spatial distribution of microorganisms, which favor distance-dependent interactions. As the inhibition of DAPG production requires cell-to-cell contact, it may only affect a minor fraction of *P. protegens* DTU9.1 cells, and hence the overall biocontrol activity against DAPG-sensitive microorganisms may be largely unaffected. This study therefore represents a defined mode of action for bacterial consortia applied as biocontrol inoculum in a natural setting and provides a stepping-stone into the much-needed mode of action studies in closer-to-field studies. An improved understanding of the underlying signalling events affecting microbial secondary metabolism may enable designs of signal-optimized microbial biocontrol consortia that potentiates production of relevant secondary metabolites to show improved efficiency in plant protection compared to single-species biocontrol.

METHODS

Microorganisms and cultivation

Bacteria were routinely cultured in Luria-Bertani broth (LB, Merck) with appropriate antibiotics. *E. coli* was cultured by inoculating a single colony in 5 ml LB broth and incubating overnight at 37 °C with shaking (200 rpm). *Pseudomonas* were cultured by inoculating a single colony in 5 ml LB broth and incubating overnight at 30 °C with shaking (200 rpm). Plasmid cloning was performed in *Escherichia coli* CC118- λ pir. Microorganisms used in this study are summarized in the Supplementary Table 1. Primers used for cloning and verification are found in the Supplementary Table 2.

Tagging *Pseudomonas* with fluorescent transposons for visualization of coculture dynamics

To visualize the development of bacterial cocultivation on solid agar surfaces, *P. protegens* DTU9.1 and *P. capeferrum* F8 were chromosomally tagged with Tn7 transposons carrying a constitutively expressed *gfp* or *mKate* gene, respectively. The plasmid, pBG42-*gfp*, was a kind gift from Victor de Lorenzo [49]. The *mKate*-version was cloned by replacing *gfp* with a PCR-amplified *mKate* gene from phymKate [50] with BclI/HindIII overhangs. The cloned plasmid, pBG42-*mKate*, was verified by Sanger sequencing at Eurofins Genomics.

The plasmids were mobilized into *Pseudomonas* by quad-parental mating and the Tn7 transposon was subsequently integrated chromosomally downstream of *glmS* in a transposase-dependent manner. For the quad-parental mating the optical density at OD₆₀₀ was normalized to 1.0 for *E. coli* HB101 harboring pRK600, *E. coli* CC118 λ pir harboring pTNS2, *E. coli* CC118 λ pir harboring pBG42-*gfp*/-*mKate* and the recipient *Pseudomonas*. Then, the bacterial suspensions were mixed in a 1:1:1:1 ratio, concentrated 50 times and finally spotted on an LB agar plate incubated at 30 °C O/N. Transconjugants were selected on *Pseudomonas* Isolation Agar (PIA, Merck) supplemented with 50 μ g ml⁻¹ gentamycin and confirmed to be expressing *gfp* or *mKate* constitutively by examination under a fluorescence microscope.

Cocultivation of *P. protegens* DTU9.1 and *P. capeferrum* F8 on solid surface

For bacterial cocultivations, 1 ml samples of *Pseudomonas* O/N cultures were washed twice in 0.9% NaCl by centrifuging at 10,000 rpm for 1 min, followed by discarding the supernatant and resuspending the pellet in fresh liquid. Subsequently, the optical density at OD₆₀₀ was normalized to 1.0. For cocultivations on agar surfaces, the bacterial suspensions of *P. protegens* DTU9.1 and *P. capeferrum* F8 were mixed in a 1:1 ratio, followed by spotting on KBmalt agar (20 g l⁻¹ Proteose peptone No. 3,

1.5 g l⁻¹ K₂HPO₄, 1.5 g l⁻¹ MgSO₄, 7.5 g l⁻¹ malt extract, 10 ml l⁻¹ glycerol and 20 g l⁻¹ Bacto agar).

Visualization and analysis of fluorescent colonies

Fluorescent colonies were imaged on a Zeiss Axio Zoom V16 stereomicroscope with a Zeiss CL 9000 LED Light source, an AxioCam 503 Monochrome camera and a PlanApo Z 0.5x/0.125 FWD 114 mm objective. The filter sets used were: Zeiss 38 HE eGFP (Ex: 470/40 nm, Em: 525/50 nm) and Zeiss 63 HE (Ex: 572/25 nm, Em: 629/62 nm). Exposure time and focus were manually optimized for each experiment, but kept identical for every sample in a given experiment. Images were acquired with Zeiss Zen and analyzed in ImageJ v1.53 [51]. Initially, the colony of interest was segmented by automatic thresholding with the Triangle method on the bright field channel with a Gaussian blur (radius = 2.00 pixels). Relative fluorescence intensity per area was measured within the resulting region of interest outlining the entire colony.

Construction of pSEVA237::P_{ptl}L-gfp reporter fusion

To investigate the effect of the secreted signal from *P. capeferrum* F8 on the transcription of the pyoluteorin biosynthetic gene cluster, a P_{ptl}L reporter fusion was constructed in pSEVA237_{RBS30} with a strong ribosomal binding site (RBS30). The promoter region was PCR-amplified with BamHI/HindIII overhangs and ligated with pSEVA237_{RBS30}. The cloned reporter fusion was verified by Sanger sequencing at Eurofins Genomics.

The plasmid was subsequently mobilized into *P. protegens* DTU9.1 by tri-parental mating. Initially, the optical density at OD₆₀₀ was normalized to 1.0 for *E. coli* HB101 harboring pRK600, *E. coli* CC118 λ pir harboring pSEVA237::P_{ptl}L-gfp and the recipient *Pseudomonas*. Then, the bacterial suspensions were mixed in a 1:1:1 ratio, concentrated 50 times and finally spotted on an LB agar plate incubated at 30 °C O/N. A transconjugant was selected on PIA supplemented with 50 µg ml⁻¹ kanamycin and confirmed to be expressing gfp by examination under a fluorescence microscope.

Distance assay

A distance assay was carried out to determine if *P. capeferrum* F8 produced a single molecule affecting both DAPG and pyoluteorin production in *P. protegens* DTU9.1. Initially, *P. capeferrum* F8 was normalized to OD₆₀₀ = 1.0 and spotted in 20 µl on KBmalt agar plates. The plates were incubated at 30 °C for 48 h. Then, a culture of *P. protegens* DTU9.1 was normalized to OD₆₀₀ = 1.0 and spotted in 5 µl with varying distance to the edge of the *P. capeferrum* F8 colony (5, 12.5 and 20 mm). As a control, *P. protegens* DTU9.1 was spotted in isolation on a separate plate. The plates were incubated at 30 °C for 24 h, followed by agar plug extraction for HR-LCMS. The decay of pyoluteorin production as a function of distance to the edge of the *P. capeferrum* F8 colony was modelled with a one phase decay function in GraphPad Prism v8.3.0, where Y₀ represents the intercept and K represents the decay rate.

$$Y = (Y_0 - \text{Plateau}) \cdot e^{-K \cdot X} + \text{Plateau}$$

Additionally, distance assays were conducted to evaluate the effect of secreted molecules on the transcription of the pyoluteorin biosynthetic genes measured as P_{ptl}L promoter activity. Similar to above, a culture of *P. protegens* DTU9.1 harboring pSEVA237::P_{ptl}L-gfp normalized to OD₆₀₀ = 1.0 was spotted in 5 µl with varying distance to the edge of a pre-cultured colony of an interacting bacterium. After 24 h of incubation at 30 °C, colonies were analyzed by fluorescent microscopy on a Zeiss Axio Zoom V16 stereomicroscope as described above.

Supernatant assay

To evaluate if *P. capeferrum* F8 secreted a signal capable of inducing transcription of the pyoluteorin biosynthetic genes in *P. protegens* DTU9.1, a supernatant assay was conducted. Initially, *P. capeferrum* F8 was cultivated for 48 h in 100 ml KBmalt broth at 30 °C with shaking (200 rpm). Supernatant was harvested by centrifuging the culture at 8,000 rpm for 5 min followed by sterile-filtration. Aliquots of the supernatant were either boiled for 60 or 120 min at 100 °C, autoclaved for 15 min at 121 °C or treated with Proteinase K (New England Biolabs) at a final concentration of 30 U/ml for 1 h at 56 °C. Then, *P. protegens* DTU9.1 harboring pSEVA237::P_{ptl}L-gfp was inoculated to an initial OD₆₀₀ = 0.001 in 50% v/v fresh KBmalt broth and supernatant (treated or untreated). Growth and fluorescence was measured every 30 min during incubation at 30 °C and continuous shake for 24 h in a Cytation5 microplate reader.

Additionally, *P. protegens* DTU9.1 was inoculated to an initial OD₆₀₀ = 0.001 in 50% v/v fresh KBmalt and sterile-filtered supernatant of *P. capeferrum* F8 or 100% fresh KBmalt (control). Tubes were incubated at 30 °C with shaking (200 rpm) for 24 h, followed by sterile filtration of the supernatant. Levels of DAPG and pyoluteorin were analyzed with HR-LCMS.

Cell lysate assay

To investigate the effect of cell-associated metabolites from *P. capeferrum* F8 on the metabolite production in *P. protegens* DTU9.1 a cell lysate assay was performed. Firstly, the cell lysate was prepared by lysis of cell pellet from 4 ml O/N culture of *P. capeferrum* F8 with NEB gDNA Tissue Lysis Buffer (New England Biolabs) and lysozyme from chicken egg-white (Sigma-Aldrich). Cell-associated metabolites were extracted with ethyl:acetate (EtOAc) extraction and resuspended in methanol to a concentration of 1 mg/ml. Then, *P. protegens* DTU9.1 was inoculated to an initial OD₆₀₀ = 0.001 in 10 ml KBmalt broth supplemented with 50 µl methanol (control) or 50 µl cell lysate extract. Tubes were incubated at 30 °C with shaking (200 rpm) for 24 h, followed by sterile filtration of the supernatant. Levels of DAPG and pyoluteorin were analyzed with HR-LCMS.

Deletion of *pltM* in *P. protegens* DTU9.1

To obtain a strain of *P. protegens* DTU9.1 incapable of synthesizing pyoluteorin, *pltM* was deleted by allelic replacement according to Hmelo et al. [52]. In short, DNA fragments directly upstream and directly downstream of the gene of interest were PCR amplified. The fragments were joined by splicing-by-overlap extension PCR with XbaI/SacI overhangs. The purified PCR product was restriction-digested and inserted in pNJ1 [53]. The resulting plasmid, pNJ1-*pltM*-del, was mobilized into *P. protegens* DTU9.1 via triparental mating with *E. coli* HB101 harboring the helper plasmid pRK600. Merodiploid transconjugants were initially selected on PIA supplemented with 50 µg ml⁻¹ tetracycline. A second selection was performed on NSLB agar (10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract, 15 g l⁻¹ Bacto agar) with 15% v/v sucrose. Candidates for successful deletion were confirmed by PCR and verified by Sanger sequencing at Eurofins Genomics.

Detection of DAPG with a whole-cell biosensor

For biosensor-guided detection of DAPG production, 1 ml O/N culture of *E. coli* CC118 λ pir harboring pSEVA225::DAPG_{lacZ} was washed twice in 0.9% NaCl and its optical density at OD₆₀₀ set to 0.5. Then, 100 µl of the biosensor suspension was spread on KBmalt plates supplemented with 65 µg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal, Thermo Fisher Scientific). *Pseudomonas* mono- and cocultures normalized to OD₆₀₀ = 1.0 were spotted on top as 20 µl colonies. All agar plates were incubated at 30 °C.

Detection of secondary metabolites with HR-LCMS

To extract secondary metabolites of mono- and cocultivated *Pseudomonas*, an agar plug covering the bacterial colony (6 mm diameter) was transferred to a vial and extracted with 1 ml of isopropanol:ethyl acetate (1:3, v/v), containing 1% formic acid, under ultrasonication for 60 min. Extracts were subsequently evaporated under N₂ and re-dissolved in 200 µl of methanol for further sonication for 15 min. Lastly, the samples were centrifuged at 13400 rpm for 3 min and supernatants transferred to HPLC vials and subjected to ultra high-performance liquid chromatography-high resolution electrospray ionization mass spectrometry analysis (abbreviated as HR-LCMS).

HR-LCMS was performed on an Agilent Infinity 1290 UHPLC system. Liquid chromatography of 1 µl or 5 µl extract was performed using an Agilent Poroshell 120 phenyl-C₆ column (2.1 × 150 mm, 1.9 µm) at 60 °C using CH₃CN and H₂O, both containing 20 mM FA. Initially, a linear gradient of 10% CH₃CN/H₂O to 100% CH₃CN over 10 min was employed, followed by isocratic elution of 100% CH₃CN for 2 min. Then, the gradient was returned to 10% CH₃CN/H₂O in 0.1 min and finally isocratic condition of 10% CH₃CN/H₂O for 1.9 min, all at a flow rate of 0.35 min ml⁻¹. HRMS data was recorded in positive ionization on an Agilent 6545 QTOF MS equipped with an Agilent Dual Jet Stream electrospray ion (ESI) source with a drying gas temperature of 250 °C, drying gas flow of 8 min l⁻¹, sheath gas temperature of 300 °C and sheath gas flow of 12 min l⁻¹. Capillary voltage was 4000 V and nozzle voltage was set to 500 V. HRMS data was processed and analyzed using Agilent MassHunter Qualitative Analysis B.07.00. HPLC grade solvents (VWR Chemicals) were used for extractions while LCMS grade solvents (VWR Chemicals) were used for LCMS.

Inhibition assay

The effect of cocultivating *P. protegens* DTU9.1 and *P. capeferrum* F8 on bioactivity towards known phytopathogenic bacteria was investigated in an inhibition assay. Cultures of *P. protegens* DTU9.1 wild type and Δ pltM mutant, as well as *P. capeferrum* F8 were normalized to an OD₆₀₀ = 1.0. Mono- and cocultures were spotted in 10 µl on KBmalt agar plates and incubated for 48 h at 30 °C, followed by bacterial inactivation by exposure to chloroform vapors for 30 min. Then, 6 ml 0.5% soft KBmalt agar was inoculated with the phytopathogenic bacteria, *Pectobacterium carotovorum* ATCC 15713 and *Dickeya solani* LMG 25993, to an OD₆₀₀ = 1.0 and added to the plates with inactivated *Pseudomonas* colonies. Plates were incubated for another 24 h at 30 °C, followed by measuring the size of inhibition zones surrounding each *Pseudomonas* colony.

Genome sequencing

Genomic DNA from *P. capeferrum* F8 was purified with the Monarch HMW DNA Extraction Kit for Tissue (New England Biolabs) according to the manufacturer's instructions. Long reads were generated with a Nanopore MinION instrument using the R9/FLO-MIN106 flow cell, while short paired-end reads were generated on the NovaSeq 6000 platform with 2x150-bp reads (Illumina). The long reads were used to *de novo* assemble the genome with Flye v2.8.1. The assembled genome was subsequently polished with the forward Illumina reads using Racon v1.4.13. The assembled genome was uploaded to and annotated by NCBI Genbank (Accession number: CP099575).

DATA AVAILABILITY

All data are included in the article and the supplemental material will be made available upon request to the corresponding author. Additionally, the *de novo* assembled genome sequence of *P. capeferrum* F8 generated in this study is available in the NCBI GenBank under the accession number CP099575.

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AUTHOR CONTRIBUTIONS

MLH and LJ conceived and designed the study. MLH carried out microbiological, molecular and genetic assays. MW and SAJ carried out metabolite extractions and chemical analyses. MW, SAJ and TOL supervised interpretation of LCMS data. MLH and LJ wrote the manuscript, with contributions and approval of all authors.

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COMPETING INTERESTS

The authors declare no competing interests.

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