1 *Pseudomonas orientalis* F9 pyoverdine, safracin and phenazine mutants remain 2 effective antagonists against *Erwinia amylovora* in apple flowers 3 

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Abstract
The recently characterized strain *Pseudomonas orientalis* F9, an isolate from apple flowers in a Swiss orchard, exhibits antagonistic traits against phytopathogens. At high colonisation densities, it exhibits phytotoxicity against apple flowers. *P. orientalis* F9 harbours biosynthesis genes for the siderophore pyoverdine as well as for the antibiotics safracin and phenazine. To elucidate the role of the three compounds in biocontrol we screened a large random knockout library of *P. orientalis* F9 for lack of pyoverdine production or *in vitro* antagonism. Transposon mutants that lacked the ability for fluorescence carried transposons in pyoverdine production genes. Mutants unable to antagonise *E. amylovora* in an *in vitro* double layer assay, carried transposon insertions in the safracin gene cluster. As no phenazine transposon mutant could be identified using the chosen selection criteria we constructed a site directed deletion mutant. Pyoverdine-, safracin-, and phenazine mutants were tested for their ability to counteract the fire blight pathogen *Erwinia amylovora* *ex vivo* on apple flowers or the soil-borne pathogen *Pythium ultimum* *in vivo* in a soil microcosm. In contrast to some *in vitro* assays, *ex vivo* and *in vivo* assays did not reveal significant differences between parental and mutant strains in their antagonistic activity. This suggests that, *ex vivo* and *in vivo*, other factors, such as competition for resources or space, are more important for successful antagonism of *P. orientalis* F9 against phytopathogens in the performed assays than the tested antibiotics or pyoverdine.
Importance

*Pseudomonas orientalis* F9 is an antagonist of the economically important phytopathogen *Erwinia amylovora*, the causal agent of fire blight in pomme fruit. On King's B medium *P. orientalis* F9 produces a pyoverdine siderophore and the antibiotic safracin. *P. orientalis* F9 transposon mutants lacking these factors fail to antagonise *E. amylovora* depending on the *in vitro* assay. On isolated flowers and in soil microcosms, however, pyoverdine-, safracin-, and phenazine mutants control phytopathogens as clearly as their parental strain.
Introduction

The ongoing concerns over the application of pesticides in plant protection intensify the need for management strategies that include the use of antagonists. For their optimal selection and use in agriculture an understanding of their mode of action is mandatory. Recently, the antagonistic activity of *P. orientalis* F9 an isolate from apple flowers in a Swiss orchard was tested against several phytopathogenic strains *in vitro* and *ex vivo* (1). *P. orientalis* F9 induced *in vitro* growth deficiency in the fire blight pathogen *E. amylovora* CFBP1430\(^{Rst}\) (*E. amylovora\(^{Rst}\)*). The fire blight pathogen affects apple, pear, and quince and is a major threat to fruit production (2). In 2016, the most effective control in fire blight management, the antibiotic streptomycin, was banned from field applications in Switzerland due to possible rise and spread of resistant pathogens (3, 4). Antagonists are a desirable alternative to antibiotics given that their efficacy in reduction of blossom infection is comparable to the most effective controls and regardless of the environmental condition, e.g. the reduction in blossom infection with biological treatments in experiments conducted between 2001 and 2007 ranged from 9.1 to 36.1\% whilst the control with streptomycin from 59 to 67.3\% (5). Besides *E. amylovora*, *P. orientalis* F9 also reduced growth of plant pathogens belonging to pathovars of *P. syringae* (*P. syringae* pv. *syringae* ACW, *P. syringae* pv. *actinidiae* ICMP 9617, and *P. syringae* pv. *persicae* NCPPB 2254) in an *in vitro* double layer assay. However, *ex vivo* on apple flowers *P. orientalis* F9 revealed phytotoxic properties when inoculated in high dose (1).

The genome of *P. orientalis* F9 contains genes for the synthesis of pyoverdine (siderophore) and synthesis genes for the antibiotics safracin and phenazine-1-carboxylic acid. Phenazines have an antibiotic activity against bacteria, fungi, and eukaryotes. They are known to successfully suppress soil-borne pathogens (6–8).
Indeed, when tested in the cress assay F9 revealed an antagonistic activity against the soil-borne pathogen \textit{Pythium ultimum} (1). Safracin belongs to the tetrahydroisoquinoline (THIQ) alkaloids, known for their broad-spectrum antibacterial activities (9) and especially strong antitumor activities. The biosynthetic gene clusters of six THIQ antibiotics have been characterized including ET-743 that has been commercialized as anticancer drug (10). Siderophores are iron chelators that bacteria produce to scavenge iron in iron deficient environments. Pyoverdines are the fluorescent pigments produced by \textit{Pseudomonas} species and their primary siderophore (11, 12). Siderophores and antibiotics have both been shown to be involved in antagonistic activities against plant pathogens (11, 13). In the presented study, we analysed the antagonistic traits of \textit{P. orientalis} F9 with regard to the siderophore pyoverdine and the antibiotics safracin and phenazine for further understanding and selection of appropriate antagonists.

Transposon mutagenesis and subsequent selection criteria (fluorescence on iron limited KB medium, halo induction of \textit{E. amylovora} in a double layer assay) led to the identification of pyoverdine – and safracin mutants. No phenazine transposon mutant was selected. To exclude the possibility of phenazine as a major player in the antagonistic traits of \textit{P. orientalis} F9, a site directed mutant of the phenazine cluster of F9 was constructed.

\textit{P. orientalis} F9 transposon - and site directed mutants were analysed for their antagonistic activity in order to correlate phenotypic traits of the mutants and their ability to counteract phytopathogens.
Results

Selected transposon mutants and site of transposon integration

The genome of *P. orientalis* F9 (GenBank accession number CP018049.1) is 5.99 Mbp in size, with an average GC content of 60.4% and no plasmids. Genome analysis revealed that the genome carries a safracin production cluster, a phenazine-1-carboxylate operon (*phzABCDEFG*, Fig. 1), and pyoverdine synthesis genes (1).

*P. orientalis* F9 was subjected to random insertion transposon mutagenesis, aiming to mutagenize potential antibiotics and siderophore genes. F9 transposon mutants were screened using double layer technique on KB agar seeded with *E. amylovora*<sup>Ref</sup>. Mutants were selected based on their inability to cause growth inhibition halos of the pathogen or on the absence of fluorescence when grown on KB plates. The transposon mutants were grouped into two phenotypic groups: no fluorescence / halo induction (nF/H) and fluorescence / no halo induction (F/nH) (Fig. 2).

The insertion site of the transposon on the *P. orientalis* F9 chromosome was determined for 15 mutants of group 1 (nF/H) and four mutants of group 2 (F/nH) using arbitrary PCR. All nF/H mutants carried the transposons within the 19 genes predicted to be part of the pyoverdine synthesis cluster and F/nH mutants within the ten genes with high similarity to the safracin cluster in *P. fluorescens* A2-2 (9).

Nine of the (nF/H) selected transposon mutants carried their transposon in Bop93_18020, which encodes for a protein 91% amino acid identical to PvdL, a pyoverdine chromophore precursor synthetase of the fluorescent bacterium *Pseudomonas synxantha* BG33R. Transposon mutants TM8, TM14, TM15, TM16 and TM17 shared the same insertion site in Bop93_18020, possibly due to clonal origin, whilst TM13 and TM38 carry their transposon closer to the 3′prime end of the gene (Fig. 3A). Mutant TM10 (nF/H, not shown) carries the transposon in BOP93_10425 a
gene with homology to \textit{pvdF} encoding the pyoverdine synthetase of \textit{Pseudomonas protegens} Pf-5 (PFL\_4090).

TM18 (F/nH) carries an insertion in Bop93\_17405. Bop93\_17405 encodes for a protein that is more than 78\% amino acid identical to SfcA which is part of the safracin cluster in \textit{P. fluorescens} ATCC 13525. TM19 (F/nH) is a transposon mutant of Bop93\_17410 which is also part of the safracin cluster (\textit{sfcB}, Fig. 3B, table 1). An additional F/nH mutant was detected with the transposon insertion in Bop93\_17440 which encodes for a protein that has identity to SfcH (85\%). For subsequent analyses in various assays, TM16 and TM18 as representative of each phenotypic group were selected.

No transposon mutant with an insertion in the phenazine gene cluster was identified. Thus, a site directed mutagenesis was performed leading to a 2.2 kb deletion in the phenazine operon of \textit{P. orientalis} F9\Delta phen::Kan\textsuperscript{R}. The mutant strain showed fluorescence when grown on KB plates and \textit{E. amylovora} halo induction in the double layer assay (F/H), (Fig. 1 and 2, table 1).

**Growth of \textit{P. orientalis} F9 and corresponding mutants in PSTB and KB medium**

The growth of \textit{P. orientalis} F9 and its mutants was evaluated in PSTB medium (14), which mimics the nutrient composition on the stigma, and in iron limited KB medium. In PSTB medium TM16 showed less growth when compared to TM18, F9\Delta phen::Kan\textsuperscript{R} or the parental strain. In KB medium all strains grew equally well (Fig. 4). When tested on siderophore indication (CAS) agar all strains including TM16 showed the same sized halo (Fig. S1). CAS phenotype and growth of TM16 in KB medium indicates an additional siderophore system present in \textit{P. orientalis} F9. However, genome analysis of F9 failed to identify additional secondary siderophores (yersiniabactin, pyochelin, achromobactine, PDTC, thioquinolobactin).
Ability of F9 transposon mutants to inhibit growth of phytopathogens and antagonists in vitro

A previous study demonstrated that *P. orientalis* F9 is capable of inhibiting the growth of bacterial phytopathogens and antagonists *in vitro* (1). Strains *E. amylovora*<sup>RM</sup>, *E. amylovora* antagonist *Pantoea vagans* C9-1, *P. syringae* pv. *syringae* ACW, *P. syringae* pv. *actinidiae* ICMP 9617, and *P. syringae* pv. *persicae* NCPPB 2254 (15–17) were poured into the top layer of a double layer assay. The assay revealed that the non-fluorescent TM16 mutant (nF/H) and *P. orientalis* F9Δphen::Kan<sup>R</sup> (F/H) induce growth deficiency similar to parental strain *P. orientalis* F9. The fluorescent but safracin negative mutant TM18 (F/nH), on the other hand, had no impact on the growth of the tested strains (Fig. 5).

Ability of F9 transposon mutants to inhibit growth of *P. ultimum* in the cress assay

The cress assay was performed with *P. orientalis* F9 and mutant strains. As shown previously (1), *P. orientalis* F9 was similarly effective as the established antagonist *P. protegens* CHA0 (Fig. 6B) when cress is co-inoculated with the soil-borne pathogen *P. ultimum* in soil. *P. orientalis* F9 and its mutants were applied onto soil containing *P. ultimum* and cress. Transposon mutants TM16, TM18, and *P. orientalis* F9Δphen::Kan<sup>R</sup> exhibited no statistically significant difference compared to the parental strain (Fig. 6A and B).
Growth inhibition of *E. amylovora*<sup>Rif</sup> mediated in an *in vitro* competition assay using stigma based medium

*P. orientalis* F9 originates from apple flowers and despite its phytopathogenic traits in the flower, the strain also significantly reduces *E. amylovora*<sup>Rif</sup> CFU on flowers. To elucidate whether or not siderophore and safracin deficiency impact on antagonistic activity *in vitro*, a PSTB competition assay was performed. PSTB medium mimics nutrients present on the stigma. *E. amylovora*<sup>Rif</sup> was co-inoculated with *P. orientalis* F9 or transposon mutants. *E. amylovora*<sup>Rif</sup> CFU were determined after three and four days of incubation. In contrast to results from the KB double layer assay, the non-fluorescent mutant TM16 revealed a reduced antagonistic activity against the fire blight pathogen, which is in accordance with PSTB growth curve results (Fig. 4B), where TM16 showed a reduced growth. There was no significant difference in the CFU of *E. amylovora*<sup>Rif</sup> co-inoculated with *P. orientalis* F9 or the safracin mutant TM18 (Fig. 7).

Inhibition of *E. amylovora*<sup>Rif</sup> by *P. orientalis* F9 mutants in the detached flowers assay

Flowers are the primary location in which *E. amylovora* replicates and subsequently invades the plant host tissue. Thus, growth reduction of *E. amylovora* in the flowers is one of the most important tasks of fire blight management. To evaluate the impact of pyoverdine, phenazine and safracin on the antagonistic activity of *P. orientalis* F9 against *E. amylovora*<sup>Rif</sup> in the apple flower, a detached flower assay was performed. *P. orientalis* F9 and transposon mutants TM16 (nF/H), TM18 (F/nH) as well as deletion mutant *P. orientalis* F9<sub>Δphen::Kan<sup>R</sup></sub> were co-inoculated with *E. amylovora*<sup>Rif</sup> onto the hypanthium of apple flowers. After two and four days of incubation, *E. amylovora*<sup>Rif</sup> was re-isolated from the apple flowers and the CFUs were determined on selective medium.
No significant difference of the determined CFUs could be detected (Fig. 8A and B).

This was also true for alternative transposon mutants TM13 (nF/H), TM19 (F/nH), and TM10 (nF/H, Fig. 8C and D).

Thus, in the detached apple flower assay, the parental *P. orientalis* F9 strain and all tested mutants had the same antagonistic activity against the fire blight pathogen. In addition, when *P. orientalis* F9, TM16, TM18 and *P. orientalis* F9Δphen::Kan<sup>R</sup> were inoculated solely with high inoculum in apple flowers, all strains revealed a phytotoxic effect (Fig. S2). Thus, in the performed detached flower assay neither the siderophore nor the tested antibiotics are major participants in antagonistic traits or necrosis of apple flowers.
Discussion

P. orientalis F9 has been shown to reduce growth of phytopathogenic microorganisms in vitro and ex vivo (1). We analyzed the impact of the strain’s antibiotics safracin and phenazine as well as its siderophore pyoverdine on its antagonistic traits.

The main siderophores produced under iron limiting conditions by fluorescent pseudomonads, including P. orientalis F9, are pyoverdines. Thus, transposon mutants of P. orientalis F9 were selected according to their loss of fluorescence on iron limited KB plates, indicative for the absence of pyoverdine. Additionally, mutants that exhibited loss of growth halo induction were selected using a double layer assay with E. amylovoraRif seeded as indicator strain in a KB top layer. Halo inducing negative mutants were shown to carry transposon insertions within genes of the safracin operon (TM18, TM19, Fig. 3B). TM16, TM13 and TM10 represented the non-fluorescent, but halo forming phenotype with the transposon inserted in the pyoverdine synthesis genes (Fig. 3A). As no transposon insertion in the annotated phenazine synthesis genes of P. orientalis F9 could be identified, a site directed mutagenesis was performed resulting in P. orientalis F9Δphen::KanR (Fig. 1).

In the KB double layer assay non-fluorescent mutant TM16 revealed growth reduction not only of E. amylovoraRif but also strains of P. syringae pathovars and E. amylovora antagonist P. vagans C9-1 similar to the wild type. The same was true for P. orientalis F9Δphen::KanR (Fig. 5). In contrast, fluorescent mutant TM18 with the transposon positioned in the stcA homolog of F9, was incapable of inducing a halo in any of the seeded strains (Fig. 5). This indicates that the production of safracin is sufficient to negatively impact in this assay on growth of the tested strains. Therefore, the antagonistic activity of P. orientalis F9 in the double layer assay can be attributed to the production of safracin but not to pyoverdine or phenazine. In contrast, when
tested in an *in vitro* competition assay in PSTB medium, which mimics the nutrient composition of the stigma (14), only the siderophore negative mutants TM16 (Fig. 7) and TM13 (data not shown) failed to reduce the CFU of *E. amylovora*<sup>Rif</sup>. This is in correspondence with the weaker growth of TM16 in PSTB medium when compared to the other strains (Fig. 4).

When tested in the *in vivo* cress assay and in the *ex vivo* detached apple flower assay the *P. orientalis* F9 mutants have the same antagonistic activity against *E. amylovora*<sup>Rif</sup> and the model soil pathogen *P. ultimum* as the parental strain (Fig. 6 and 8). The CFUs of *E. amylovora*<sup>Rif</sup> recovered after co-inoculation with *P. orientalis* F9, corresponding transposon mutants TM16, TM18, alternative transposon mutants TM13, TM10 and TM19, or *P. orientalis* F9Δphen::Kan<sup>R</sup> into apple flowers did not differ significantly (Fig. 8). The same was true for the cress biomass defined after co-inoculation of the pathogen with parental or mutant strains (Fig. 6). *E. amylovora* produces the hydroxamate siderophore desferrioxamine E (DFO E) (18–20). The importance of this system for the pathogenicity of *E. amylovora* has been demonstrated using mutants deficient in siderophore synthesis or uptake (21). The lack of pyoverdine production in TM16 and the subsequent reduced competition of the mutant strain for iron in the apple flower was expected to impact on its ability to counteract *E. amylovora*. Thus, it was unexpected, that TM16 was still able to significantly reduce *E. amylovora*<sup>Rif</sup> in the detached flower assay. When poured on CAS agar TM16 produce a halo (indicative for siderophore synthesis, Fig. S1) equal in size to that of parental strain F9. Genome analysis could not reveal additional synthesis genes for secondary siderophores such as yersiniabactin, pyochelin, achromobactine, PDTC, or thioquinolobactin. Either F9 carries an unidentified system or the CAS assay interferes with alternative iron chelating agents e.g. citrate that triggered the CAS signal in case of *Bradyrhizobium*
japonicum and enabled the strain to uptake iron (22). In case of the marine pathogen
Photobacterium damselae subsp. damselae a mutant lacking the citrate synthase
(GltA) showed almost no reaction in the CAS test (23). The P. orientalis F9
chromosome encodes for a protein with 72.5% identity. In addition, phenazine-1-
carboxyclic acid production has also an impact on iron availability e.g. strains
producing the antibiotic can have an enhancing effect on the reactivity and mobility of
iron derived from soil minerals (24). The reason why the results of the double layer
assay and competition in PSTB liquid medium contradict each other is unknown.
Potentially, the experimental conditions in the two assays select for different
transcriptional activities in P. orientalis F9. Siderophore production is regulated by Fe²⁺
concentration in the cell (25, 26), while the regulation of safracin production is
unknown. Generally, antibiotic production has been shown to be regulated via quorum
sensing, but also by presence and absence of interspecies competition (27, 28). Genes
coding for proteins with high similarity to the LasI/R and RhlI/R QS systems that
regulate the production of multiple virulence factors in P. aeruginosa (29) could not
be detected in P. orientalis F9.
Safracin has been shown to be a broad-spectrum antibiotic (30) and indeed caused
strong inhibition in vitro. However, it appears to have a minor or no role in competition
against E. amylovoraRif in detached flowers or against P. ultimum in soil. There are
additional metabolites that are major candidates for the antagonistic performance of
P. orientalis F9 in the in vivo assays. The strain also harbours poaeamide
(BOP93_16455) and obaflurin synthesis genes on its genome. Poaeamide inhibited
mycelial growth of R. solani and different oomycetes, including P. ultimum (31). This
might explain why the phenazine deletion mutant of F9 is still able to antagonize
P. ultimum in the cress assay. The β-lactone antibiotic obafluorin produced by
P. fluorescens ATCC showed a weak antibacterial activity against a range of bacteria by disk diffusion (32). In addition, P. orientalis F9 also harbours genes with homology to the Hcp secretion island-1 encoded type VI secretion system (H-T6SS, BOP93_RS26545 to BOP93_RS26640). In case of Serracia marcescens Db10 the T6SS exhibit antibacterial killing activity (33). Future analyses have to show if these features of P. orientalis F9 are major players in the strain’s repertoire of antagonistic traits in the apple blossom or cress assay.

Results presented in this paper demonstrate that competition and antagonism is multifactorial and not solely dependent on antibiotic and / or siderophore production. Investigating the causal reason for the different results is however out of the scope of this study. The actual cause for antagonism is thus still unclear and could be mediated by the plant host, resource competition, or the antibiotics that have not been studied (28, 34, 35). Our results highlight the importance of a proper choice of screening systems that need to be sufficiently close to environmental conditions. Results of in vitro screens do not reflect in situ results and need to be supplemented by additional corroborative assays.
Material and Methods

Cultivation of bacterial strains used

Bacterial overnight cultures were grown at 26 °C in Tryptic Soy broth (TSB, Oxoid, Karlsruhe, Germany) or King's B medium (KB, (36). PSTB medium (14) (3 g K₂HPO₄, 1 g NaH₂PO₄, 0.39 g MgSO₄, 1.2 g niacin, 0.8 g NH₄Cl, 0.2 g amino acid mix (proline, asparagine, glutamine and serine in ratio of 3:2:2:1), 25 g glucose and 25 g fructose L⁻¹). Microorganisms used in the study are listed in table 1. Where appropriate, media was supplemented with kanamycin or rifampicin at a concentration of 40 mg L⁻¹ or 100 mg L⁻¹, respectively.

Transposon mutagenesis

Random insertion Tn10 transposon mutagenesis was performed using pJA1, an oriR6K-based suicide vector. The plasmid contains the Tn10 transposase and confers kanamycin resistance (37). Donor E. coli SM10 λpir (pJA1) and recipient P. orientalis F9 were grown overnight in either LB (Carl Roth, tryptone 10 g L⁻¹, yeast extract 5 g L⁻¹, NaCl 10 g L⁻¹, pH 7.0 ±0.2) supplemented with kanamycin or KB, respectively. The overnight culture of E. coli SM10 λpir (pJA1) was inoculated into fresh LB containing kanamycin and grown to mid exponential phase. One ml of the donor and the recipient in stationary phase were mixed and centrifuged for 30 sec at 10,000 g. The bacterial pellet was washed twice with 1 x phosphate buffered saline (PBS, K₂HPO₄ 2.5 g L⁻¹, KH₂PO₄ 1.2 g L⁻¹) and subsequently spotted onto the center of an IPTG-containing LB plate (10 μl of a 100 mM IPTG solution, spotted onto the center of the plate). The conjugation plate was incubated at 37 °C for 3 to 5 hours. A dilution series was plated onto MM2-agar plates (4 g L⁻¹ L-asparagine, 2 g L⁻¹ K₂HPO₄, 0.2 g L⁻¹ MgSO₄, 3 g L⁻¹ NaCl, 10 g L⁻¹ sorbitol, 15 g L⁻¹ agar) supplemented with kanamycin.
After 2 to 3 days of growth at 26 °C, single colonies were picked and inoculated in 96-well plates containing KB and grown for two days at 26 °C. *P. orientalis* F9 mutants were screened for two phenotypes: 1) a lack of fluorescence in the iron limited KB agar and 2) by the lack of growth deficiency halos on KB plates overlaid with *E. amylovora* Rif in the double-layer assay (see below).

**Identification of transposon insertion sides**

Transposon insertion sites were identified according to Holenstein (38). As previously described (39), transposon-insertion specific primers arPCR-T7 (5' GCACCTAACCGCTAGCACGTATACGACTC-3') and ARB6 (5' GGCCACGCCTCGACTAGTACNNNNNNNNACGC-3') were used for a primary PCR. In a second, nested, PCR primers arPCR-T7 inner (5' TGAACGGTAGCATCTTGACGAC-3') and ARB2 (5' GGCCACGCCTCGACTAGTACGNACGCC-3') were used. A crude DNA extract of selected transposon mutants was prepared by suspending 24-48 h old bacterial colonies in 300 µl double-distilled H$_2$O, incubated at 95 °C for 30 min and centrifuged at 12,000 g for 1 min. The supernatant was diluted 1:10 with double-distilled H$_2$O and used as PCR template. Amplifications were performed using Hotstar Taq polymerase (Qiagen). PCR products were sequenced using an ABI Prism BigDye Terminator version 1.1 cycle sequencing kit (Applied Biosystems) and analysed using nucleotide NCBI BLAST against the *P. orientalis* F9 whole genome sequence (1) and protein BLAST using the UniProt database using default settings (40).
Site-directed mutagenesis of *P. orientalis* F9

A phenazine mutant of *P. orientalis* F9Δphen::KanR was generated by site-directed mutagenesis. For this purpose, the *BglII*, *EcoRI*, and *EcoRV* cut sites within the phenazine gene cluster (see Fig. 1) were used. Two fragments of ca. 1.4 kb (primers PhenaF 5′-GTCGTGGAAGCTGGACAGTG-3′ / PhenaEVr 5′-GACTCGGCGATCCTGATTG-3′, annealing temperature 58 °C) and 1.8 kb (primers PhenaEVf 5′-TGGTGTTGCCTGCGAGGT-3′ / PhenaR 5′-AACCGGTGAACCCCTTGTTT-3′, annealing temperature 58 °C) of a 5.2 kb *BglII* / *EcoRI* fragment of the phenazine gene cluster were amplified by PCR. The 1.4 kb PCR product was digested with *BglII* / *EcoRV* the 1.8 kb product *EcoRV* / *EcoRI* respectively.

The *BglII* / *EcoRV* product was ligated in a first step into the similarly digested suicide vector pKAS32 (41), followed by ligation with the *EcoRV* / *EcoRI* -digested 1.8 kb fragment and the appropriate cut vector of the first ligation step. Resulting plasmid pSV11+2 harboured the 5′ and 3′ end flanking regions of the *BglII* / *EcoRI* phenazine fragment with a 2.2 kb deletion in between. pSV11+2 was *EcoRV* digested and ligated with a *HindIII*-cut kanamycin cassette from pSB315 (42), resulting in pSVKan. When using pKAS32 derivatives for the positive selection of a double allelic exchange, a streptomycin-resistant parental strain is required (41). Spontaneous streptomycin-resistant colonies of *P. orientalis* F9 were isolated by increasing (start concentration 10 µg/ml, final concentration 100 µg/ml) streptomycin concentrations in KB. pSVKan was conjugated into *P. orientalis* F9SmR using two-parental mating employing *E. coli* S17-1 λpir (43). Kanamycin- and streptomycin-resistant mutants were selected on MM2 medium containing 40 µg/ml kanamycin and 500 µg/ml streptomycin. Strain *P. orientalis* F9Δphen::KanR (SmR) was tested via PCR using primers for the presence of the kanamycin resistance gene (aph157 5′-GTCACCGAGGCAGTTCCA-3′ / aph606 5′-
CGACCATCAAGCATTTTATC-3’, annealing temperature 58 °C) and primers set before the EcoRV restriction site. (DelF 5’-AGGTGAACGTCTTCGGCG-3’ / DelR 5’-CTCCGATCATGTGATCCGC-3’. DelF and DelR amplified a ca. 700 bp fragment and the integrated kanamycin resistance cassette. Position of the kanamycin cassette within the former EcoRV cutting site was confirmed by sequencing.

Bacterial growth rate analysis

For growth rate analysis the Bioscreen C (Oy Growth Curves Ab Ltd, Helsinki) automatic microbiology growth curve analysis system was used. 900 μl medium (KB or PSTB) were pipetted into a reaction, 200 μl of which were not inoculated but used as a negative control for the corresponding growth curves. The remaining 700 μl were inoculated with 3 μl overnight culture of the tested strain. Three replicates of the inoculated medium, each 200 μl, were loaded in wells of a Bioscreen C honeycomb plate. The plates were incubated at 26 °C for 24 hours and were shaken every 20 min for 10 s before absorbance at OD_{600nm} was determined. Growth experiments were performed in two independent trials.

Siderophore assay

Siderophore production was tested using Chrome azurol S agar (44). Bacteria were grown overnight on TSB plates, harvested and resuspended in PBS buffer to an OD\textsubscript{600nm} = 1. Five μl of the bacterial suspension were spotted onto Chrome azurol S agar and incubated for three days at 26 °C. Siderophore-producing strains induce a colour change from a green-blue CAS-iron complex to orange desferrated CAS.
Phytotoxicity test of *P. orientalis* F9 and mutant strains

For the phytotoxicity test *P. orientalis* F9 and mutant strains were cultivated overnight on TSB plates. Colonies were resuspended in 1 × PBS and adjusted to OD$_{600nm}$ = 1. Twenty μl of a 10$^{-2}$ dilution of the suspension were applied onto the hypanthium of apple flowers. Inoculated flowers were incubated at 26 °C in closed boxes with water saturated paper towels and necrosis was evaluated four-days post infection (d.p.i.).

Growth inhibition test using the double layer assay

For the double layer assay the strains were cultivated overnight on KB plates. Colonies were resuspended in 1 × PBS and adjusted to OD$_{600nm}$ = 1. For bacteria seeded in the top layer approximately 5 × 10$^8$ bacteria were added to 10 ml of 0.75% KB top agar (cooled to 45 °C). Fifteen ml of a top agar were poured on top of a 12 cm square KB agar plate. Ten μl of each *P. orientalis* F9 mutant strain tested as antagonist were spotted onto the solidified top layer surfaces. Growth halos were detected after two days of incubation at 26 °C.

Stigma based media *in vitro* competition assay

*E. amylovora* $^{Rid}$, *P. orientalis* F9 and mutants were cultivated on KB plates overnight. Colonies were harvested and resuspended in 1 × PBS buffer to an OD$_{600nm}$ = 1 before serially diluted up to 10$^{-4}$. Twenty μl of this bacterial suspensions were then added to 980 μl 1 × PBS, or, in the case of a co-inoculation, 20 μl of each strain were added to 960 μl 1 × PBS. Sixty μl of the bacterial suspension were directly pipetted into 1440 μl of PSTB medium, vortexed and then aliquot to 400 ul in three columns of a 96 deep well plate. After three days at 26 °C the CFU of *E. amylovora* $^{Rid}$ were determined. A serial dilution of the inoculated medium was performed up to 10$^{-7}$. Three μl of each
dilution were spotted onto TSB plates supplemented with rifampicin (100 μg/ml). Experiments were repeated three times independently.

**Cress assay**

For the cress assay (45), two 1 cm diameter discs of *Pythium ultimum* culture grown on Malt extract agar (Oxoid) were punched out, laid on the bottom of a 9 cm diameter petri dish and carefully overlaid with 14 g double autoclaved soil. *P. orientalis* F9 and F9 mutants were grown on KB plates overnight and resuspended in 1 × PBS buffer to an OD$_{600nm}$ = 0.1. Ten ml of the bacterial suspensions or mock control (PBS only) were evenly spread over the soil surface, followed by 0.4 g cress seeds (*Lepidium sativum*). Petri dishes were incubated at 22 °C and 65% humidity. After two, four and six days of incubation, 20 ml of autoclaved water were added. After seven days of incubation, the complete above ground cress biomass was harvested and its wet weight determined. Each treatment was performed in duplicates for inoculation of cress only with *P. orientalis* F9, TM16, TM18, *P. orientalis* F9Δphen::Kan$^R$, and *P. protegens* CHA0 or in quadruplicates for *P. ultimum* controls and co-inoculations of *P. orientalis* F9, TM16, TM18, *P. orientalis* F9Δphen::Kan$^R$, and *P. protegens* CHA0, with *P. ultimum*. The experiments were repeated 2-3 times independently.

**Detached flower assay**

For the detached flower assay (46), freshly opened flowers of two-year-old potted *Malus domestica* ‘Golden Delicious’ in the greenhouse were used. *E. amylovora*$^{Rif}$, *P. orientalis* F9 and mutants were cultivated and resuspended to an OD$_{600nm}$ = 1 as described above. A serial dilution of each bacterial suspension was performed. For the *E. amylovora* control 20 µl of the $10^{-4}$ dilution were transferred into 980 µl of PBS, for
co-inoculations 20 μl of each strain in 960 μl PBS. Twenty μl of the bacterial suspensions were directly pipetted onto the hypanthium of individual flowers. Mock treatments were performed with 1 × PBS. After inoculation, flowers were incubated at 26°C in closed boxes with water saturated paper towels on the bottom. Two- and four-days post infection (d.p.i.), the colony forming units (CFU) of *E. amylovora*<sup>Rif</sup> in 8 individual flowers of each treatment was determined as described previously (1).

Briefly, petals, pedestals, stamens, and stigmas of the flowers were removed and the remaining flowers shaken in 1 ml 1 × PBS buffer for 30 min at 1400 rpm. Afterwards, tubes were vortexed for 30 seconds. A serial dilution of the supernatant suspension was performed up to 10<sup>-7</sup> and 3 μl of each dilution were spotted onto TSB plates supplemented with rifampicin (100 μg/ml). Detached flower assays for transposon mutants TM16 and TM18 (and alternative mutants TM10, TM13, and TM19) were performed during spring and summer 2018, for the phenazine mutant *P. orientalis* F9Δphen::Kan<sup>R</sup> in spring 2019. Assays were repeated at least three times independently.

**Genome analysis of *P. orientalis* F9**

The genome of *P. orientalis* F9 (CP018049.1.1) was analysed for additional metabolites and factors with potential antagonistic activity using VFDB (virulence factors of bacterial pathogens) and antismash. In addition, blastn / tblastn (NCBI, national centre for biotechnology information) comparison of the *P. orientalis* F9 genome and synthesis genes for additional secondary siderophores of pseudomonads was also performed using the sequences for enantio-pyochelin (47), pseudomonine (48), pyridine-2,6-bis(monothiocarboxylic acid) (PDTC, 49), and thioquinolobactin (50), synthesis genes.
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| Strain                        | Notes                                                                 | Reference or source |
|------------------------------|----------------------------------------------------------------------|---------------------|
| *P. orientalis* F9           | isolated from *Malus domestica*, flower, canton Zurich, CH, 2014 fluorescence, halo induction (F/H) | (1)                 |
| *P. orientalis* F9 TM10      | Transposon mutant of *P. orientalis* F9, no fluorescence, halo induction (nF/H) | This study          |
| *P. orientalis* F9 TM13      | Transposon mutant of *P. orientalis* F9, no fluorescence, halo induction (nF/H) | This study          |
| *P. orientalis* F9 TM16      | Transposon mutant of *P. orientalis* F9, no fluorescence, halo induction (nF/H) | This study          |
| *P. orientalis* F9 TM18      | Transposon mutant of *P. orientalis* F9, fluorescence, no halo induction (F/nH) | This study          |
| *P. orientalis* F9 TM19      | Transposon mutant of *P. orientalis* F9, fluorescence, no halo induction (F/nH) | This study          |
| *P. orientalis* Δphen::KanR  | Phenazine mutant of *P. orientalis* F9, fluorescence, halo induction (F/H), deletion in *phzCDE* | This study          |
| *E. amylovora* CFBP1430Rif   | Spontaneous rifampicin mutant of *E. amylovora* CFBP1430               | (1)                 |
| Organism | Description |
|----------|-------------|
| *P. vagans* C9-1 | E. amylovora antagonist |
| *P. syringae pv. persicae* NCPPB 2254 | Causal agent of CFBP bacterial die-back in peach, nectarine, Japanese plum |
| *P. syringae pv. actinidiae* ICMP 9617 | Causal agent of CFBP bacterial canker of kiwifruit |
| *P. syringae pv. syringae* ACW | Causal agent of bacterial canker of pome and stone fruit |
| *P. protegens* CHA0 | Model organism in biological control of soil born pathogens |
| *E. coli* SM17-1 λpir | pir⁺, tra⁺, SmR |
| *E. coli* SM10 λpir (pJA1) | Contains the suicide vector pJA1, pir⁺, tra⁺, KanR |
| *Pythium ultimum* | Soil-born phytopathogen |

Plasmids

| Plasmid | Description |
|---------|-------------|
| pKAS32 | Cloning vector with rpsL gene, AmpR |
| pSB315 | Containing kanamycin cassette without transcriptional terminator, AmpR, KanR |
| pJA1 | Containing transposon, KanR |
Figure legends

Fig. 1: Schematic representation of the phenazine gene cluster in *P. orientalis* F9. Large grey arrows indicate coding sequences. Small black horizontal arrows show position and direction of primers used for site-directed mutagenesis. The PCR generated fragments were cut at naturally occurring EcoRV restriction sites for insertion of the kanamycin resistance cassette (Δphen::KanR) leading to deletion of a 2.2 kb fragment of the *P. orientalis* F9 phenazine cluster. Genes were assigned with their corresponding accession number from the sequenced *P. orientalis* F9 genome and correspond to the six genes *phzABCDEF* of the phenazine cluster (from right to left).

Fig. 2: Double layer assay with *P. orientalis* F9 (F9, F/H), transposon mutants TM16 (nF/H), TM18 (F/nH), and phenazine mutant *P. orientalis* F9Δphen::KanR (Δphen::KanR, F/H) pipetted onto *E. amylovora* CFBP1430 seeded in a KB over layer agar. Presence and absence of *E. amylovora* inhibition zones are visualized in the KB double layer assay, UV light reveals the presence or absence of the fluorescence that is indicative for siderophore production. nF/H: no fluorescence / halo induction; F/nH: fluorescence / no halo induction; F/H: fluorescence / halo induction.

Fig. 3A and B: Schematic representation of transposon location for each defined phenotype group. Coding sequences are indicated by large horizontal arrows which show the direction of transcription. Black vertical arrows show the integration site of transposons. Dashed lines represent intermediate DNA regions, their length stated in kb. Genes were assigned to the annotated *P. orientalis* F9 genome with their corresponding Bop number. A: Transposon location of fluorescence negative but halo
inducing mutants (nF/H) in genes Bop93_17980 and Bop93_18020. Homology study allocated the two genes within the pyoverdine gene cluster. TM16 and TM13 were used in further analysis (marked bold and underlined). B: Transposon location of fluorescent but not halo inducing mutants (F/nH). Insertion occurred in three different genes of the safracin cluster. TM18 and TM19 (marked bold and underlined) were used in further analysis.

Fig. 4: Growth curve of *P. orientalis* F9 (black line), *P. orientalis* F9 Δphen::KanR (broken line), nF/H mutant TM16 (dotted line), and F/nH mutant TM18 (dotted, broken line) in KB (A) and stigma based (PSTB) medium (B). Error bars represent standard deviations.

Fig. 5: Double layer assay: *P. orientalis* F9 and corresponding mutant strains and their impact on the growth of plant pathogens and antagonists seeded in a KB over layer. A: *E. amylovora* CFBP1430; B: *P. vagans* C9-1 (*E. amylovora* antagonist); C: *P. syringae* pv. *syringae* ACW; D: *P. syringae* pv. *actinidiae* ICMP 9617; E: *P. syringae* pv. *persicae* NCPPB 2254. Spotted on top: *P. orientalis* F9 (F9), *P. orientalis* F9 Δphen::KanR (Δphen::KanR), nF/H mutant TM16 (TM16), and F/nH mutant TM18 (TM18), not announced (n.a.).

Fig. 6 A: Cress biomass after treatment with *P. ultimum* (PU) and/or *P. orientalis* F9 (F9) and corresponding transposon mutants TM18 and TM16.

B: Cress biomass after treatment with *P. ultimum* (PU) and/or *P. orientalis* F9 (F9), *P. orientalis* F9 Δphen::KanR (Δphen::KanR) and known antagonist *P. protegens* CHA0 (CHA0). Cress biomass was harvested seven days post inoculation. Error bars
depict the standard error of the mean. Different letters depict significant differences between measurements (one-way ANOVA, multiple comparisons test with Tukey correction, P < 0.0001).

Fig. 7: In vitro competition assay of *P. orientalis* F9 (F9) and transposon mutants TM16 (nF/H), and TM18 (F/nH), co-cultivated with *E. amylovora* \(^{\text{Rif}}\) \((\text{Ea})\) in stigma based (PSTB) medium at 26 °C. *E. amylovora* \(^{\text{Rif}}\) CFU were determined after three days of incubation. Error bars represent standard deviation of the mean. Different letters depict significant differences between measurements (one-way ANOVA, multiple comparisons test with Tukey correction, P < 0.05).

Fig. 8: A, B, C, and D: Antagonistic activity of *P. orientalis* F9 and mutants against *E. amylovora* \(^{\text{Rif}}\) \((\text{Ea})\) in apple flowers. A: Recovered CFU (log) of *E. amylovora* \(^{\text{Rif}}\) control and *E. amylovora* \(^{\text{Rif}}\) co-inoculation with *P. orientalis* F9 (F9), nF/H mutant TM16 (TM16), and F/nH mutant TM18 (TM18) or B: *P. orientalis* F9Δphen::Kan\(^{\text{R}}\) (Δphen::Kan\(^{\text{R}}\)) after two and four days post infection. C: Antagonistic activity of *P. orientalis* F9 alternative mutants TM13 (nF/H) and TM19 (F/nH) and, D: TM10 (nF/H) five days post infection. Error bars represent standard deviation of the mean. Different letters depict significant differences between measurements (Two-way ANOVA, multiple comparisons test with Tukey correction, P < 0.005).
