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Plant pathogenic bacterium *Ralstonia solanacearum* can rapidly evolve tolerance to antimicrobials produced by *Pseudomonas* biocontrol bacteria

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Abstract

Soil-borne plant pathogens significantly threaten crop production due to lack of effective control methods. One alternative to traditional agrochemicals is microbial biocontrol, where pathogen growth is suppressed by naturally occurring bacteria that produce antimicrobial chemicals. However, it is still unclear if pathogenic bacteria can evolve tolerance to biocontrol antimicrobials and if this could constrain the long-term efficacy of biocontrol strategies. Here we used an in vitro experimental evolution approach to investigate if the phytopathogenic *Ralstonia solanacearum* bacterium, which causes bacterial wilt disease, can evolve tolerance to antimicrobials produced by *Pseudomonas* bacteria. We further asked if tolerance was specific to pairs of *R. solanacearum* and *Pseudomonas* strains and certain antimicrobial compounds produced by *Pseudomonas*. We found that while all *R. solanacearum* strains could initially be inhibited by *Pseudomonas* strains, this inhibition decreased following successive subculturing with or without *Pseudomonas* supernatants. Using separate tolerance assays, we show that the majority of *R. solanacearum* strains evolved increased tolerance to multiple *Pseudomonas* strains. Mechanistically, evolved tolerance was most likely linked to reduced susceptibility to orfamide lipopeptide antimicrobials secreted by *Pseudomonas* strains in our experimental conditions. Some levels of tolerance also evolved in the control treatments, which was likely correlated response due to adaptations to the culture media. Together, these results suggest that plant-pathogenic bacteria can rapidly evolve increased tolerance to bacterial antimicrobial compounds, which could reduce the long-term efficacy of microbial biocontrol.

Keywords: experimental evolution, antimicrobial resistance, plant pathogenic, bacteria, biocontrol, *Pseudomonas*

Introduction

The use of traditional chemical pesticides has declined in recent years due to cost, environmental toxicity, and legislation (Chen et al., 2016). As a result, new methods are needed to control plant pathogen outbreaks and to ensure future food security in the face of an expanding human population (Kaczmarek et al., 2014). Biocontrol is a means of using natural competitors, such as nonpathogenic microorganisms, to control the growth and limit the damage caused by plant pathogens. For example, naturally occurring soil-borne *Pseudomonas* bacteria have been shown to constrain the growth of various plant pathogenic bacteria, fungi, and nematodes (Pierson et al., 1998) through resource and interference competition via antibiosis (Hu et al., 2016; Weller, 2007). While multiple *Pseudomonas* species with biocontrol potential have been identified, it is still unclear how broadly they can inhibit the growth of different strains of one given pathogen species. Understanding the variation in pathogen susceptibility is important because plant pathogen populations are often genetically highly diverse within agricultural fields (Xue et al., 2013). If this variation is also linked to pathogen susceptibility to biocontrol agents, natural selection is likely to increase the prevalence of tolerant or resistant pathogen genotypes over time, reducing the long-term efficiency of pathogen suppression.

Biocontrol efficiency could also differ between different biocontrol species or genotypes, due to variation in their secondary metabolism (Becker et al., 2012). For example, the number of secondary metabolites produced by different *Pseudomonas* strains ranges from a variety of antimicrobials to the production of iron-scavenging siderophores (Geller & Levy, 2023). For example, the production of hydrogen cyanide (HCN) and cyclic lipopeptide antibiotics known to be active against multiple pathogens, including bacteria and fungi (Geudens & Martins, 2018; Nielsen et al., 2002), is specific to *Pseudomonas protegens* CHA0 and Pf-5 strains (Haas & Keel, 2003). In contrast, other secondary metabolites, such as the production of 2,4-diacetylphloroglucinol (DAPG), which is an effective antimicrobial against bacterial, fungal, and nematode plant pathogens (Compant et al., 2005; Cronin et al., 1997; Haas & Défago, 2005; Humair et al., 2009) is more common among different *Pseudomonas* species. It is also possible that different species or genotypes produce different amounts of secondary metabolites, which could

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further affect their biocontrol efficacy (Landa et al., 2002). The amount and type of produced antimicrobials could in turn affect the trajectory of tolerance evolution by targeted plant pathogens. For example, a causative agent of bacterial wilt disease—phytopathogenic *Ralstonia solanacearum* bacterium—has been recently shown to rapidly evolve more tolerance to allelochemicals produced by *Brassica* plants, resulting in cross-tolerance to antibiotics produced by soil bacteria (Alderley et al., 2022). As over two-thirds of the clinical antibiotics—to which bacteria have repeatedly been shown to evolve resistance to—are produced by soil bacteria (Gillespie, 2002; Kieser et al., 2000), it is likely that plant pathogenic bacteria could also evolve tolerance to antimicrobials produced by bacterial biocontrol agents.

Here we studied this by using a model system consisting of seven isolates of the plant pathogenic bacterium *R. solanacearum,* which are all closely related members of the phylotype IIB sequevar 1 potato brown rot pandemic lineage, and eight different *Pseudomonas* biocontrol bacterial strains (Supplementary Table S2). *Ralstonia solanacearum* is a globally spread quarantine pathogen which causes vascular wilt disease (EPPO, 2021). It possesses numerous virulence mechanisms that enable the efficient infection of more than 200 plant species from 50 plant families (Genin & Denny, 2012; Hayward, 1991; Mansfield et al., 2012; Nion & Toyota, 2015). *R. solanacearum* is highly variable genetically and this variation has previously been linked to its susceptibility to antimicrobials produced by different soil bacteria (Xue et al., 2013). The eight *Pseudomonas* species selected for this study have previously been shown to have a range of plant growth-promoting properties that can contribute to *R. solanacearum* pathogen suppression (Becker et al., 2012; Haas & Défago, 2005; Hu et al., 2016; Ma et al., 2016). However, it is unclear if the efficacy of *Pseudomonas* strains depends on the *R. solanacearum* strain identity. Moreover, it is not clear what are the underlying mechanisms of *R. solanacearum* inhibition by *Pseudomonas*, and if *R. solanacearum* can evolve more tolerant or resistant to *Pseudomonas* in the long-term. To study this experimentally, we exposed seven *R. solanacearum* UK strains to the supernatants of eight *Pseudomonas* species in pairwise combinations in a selection experiment that lasted for 21 days. Following the selection experiment, fitness assays were performed to explore if any strains had developed tolerance to *Pseudomonas* supernatants, tolerance is defined as a bacterial population experiencing a lower killing rate and/or growth reduction over time (Balaban et al., 2019). Moreover, microbiological assays were used to identify and test the antimicrobial effects of candidate metabolites secreted by the most inhibitory *Pseudomonas* strains. It was found that all *R. solanacearum* populations were able to overcome *Pseudomonas* inhibition over time through tolerance evolution. Together, these findings suggest that the potential risks of pathogen tolerance and resistance evolution should be considered when developing *Pseudomonas* biocontrol applications based on their antimicrobial activity.

Methods

Culturing and maintenance of bacterial strains

Eight fluorescent Pseudomonad strains (CHA0, Pf-5, Q2-87, Q8R1-96, 1M1-96, MVP1-4, F113, and Ph11C2) and seven *R. solanacearum* strains (#1–7) isolated as a part of annual river sampling survey in England and Wales were used in the experiments (listed in Supplementary Tables S1 and S2). All bacteria were stored in 20% glycerol stocks at −80 °C. Prior to experiments, bacterial starting cultures were prepared as follows: frozen samples were inoculated in 5 ml of Luria-Bertani (LB) broth (media recipe described in Supplementary Table S3) and incubated with shaking at 200 rpm at 28 °C for 24 hr. Bacterial cultures were prepared similarly throughout all experiments unless stated otherwise.

The selection experiment and quantification of pathogen population density dynamics

To investigate pathogen tolerance evolution to *Pseudomonas* antimicrobials, we exposed each *R. solanacearum* strain to the supernatants of each *Pseudomonas* species in pairwise pathogen and *Pseudomonas* supernatant combinations in a 21-day-long selection experiment. The *Pseudomonas* supernatants included all secreted secondary metabolites produced when *Pseudomonas* strains were grown alone in LB media—a method that has previously been shown *Pseudomonas* antimicrobial activity against Chinese *R. solanacearum* strain (Hu et al., 2016). *Pseudomonas* supernatants were prepared as follows. All *Pseudomonas* strains were first cultured individually in 20 ml of LB broth (Supplementary Table S3) in 50 ml falcon tubes for 24 hr at 28 °C with shaking at 200 rpm and centrifuged for 10 min at 4000 *g*. The supernatant was then filtered using a 0.2 µm filter to separate bacterial cells and fragments from secondary metabolites present in the supernatants. We used flat-bottomed 96-well plates for the selection experiments and used two supernatant-LB concentrations for each *Pseudomonas* strain: low (1:1, i.e., 50%) and high (4:1, i.e., 80%). Controls included *R. solanacearum* grown in low (1:1, i.e., 50%) and high (4:1, i.e., 80%) LB broth diluted with sterile H_2O instead of *Pseudomonas* supernatant (LB broth: H_2O). All supernatant combinations were replicated 4 times and control treatments 3 times. At the start of the experiment, supernatant and control combination were inoculated with 2 µl of each *R. solanacearum* strain (approximately 1.0×10^6 cells ml^{-1}) in 200 µl of media. Microplates were then incubated without shaking (to enable natural population structure) at 28 °C for the whole duration of the experiment and bacterial densities were measured every 72 hr as optical density (Optical Density [OD] 600 nm; Tecan Infinite spectrophotometer) after the first reading at 24 hr time point. At each time point, the plates were removed from the incubator and the optical density was measured before serial transfers. Following these measurements, subsamples of bacterial cultures (10%) were serially transferred to fresh supernatant and control treatments every 3 days after mixing replicate populations gently with pipette. All populations were cryopreserved in 20% glycerol-LB media (Supplementary Table S3) every second transfer (2nd, 4th, 6th, and 8th transfer), and the last sample was used for fitness assays.

Fitness assays for determining evolution of antimicrobial tolerance

To quantify potential evolution of antimicrobial tolerance and its associated growth costs, the relative fitness of ancestral *R. solanacearum* strains was compared with the control ("LB-exposed") and evolved ("supernatant-exposed") *R. solanacearum* populations at the final time point of the selection experiment (21-day time point, which equalled approximately 300 *R. solanacearum* generations; estimated doubling time was 90 min). Ancestral and LB-evolved

populations were grown in both the absence and presence of ancestral *Pseudomonas* supernatants and in LB broth dilutions without supernatants. The evolution of tolerance was only tested in the presence of *Pseudomonas* supernatant to which the specific pathogen strain had previously been exposed during the selection experiment. For example, if the pathogen strain had been exposed to *Pseudomonas* CHA0 strain, its tolerance was quantified only in the presence of the ancestral *Pseudomonas* CHA0 supernatant. Similarly, tolerance was measured only in the same supernatant concentration populations had been exposed to during the selection experiment (low in low and high in high). Ancestral, supernatant-exposed, and LB-exposed cryopopulations were regrown for 24 hr prior to assays in 200 µl of 100% LB broth at 28 °C. *Pseudomonas* supernatants were derived by growing ancestral *Pseudomonas* strains in 20 ml of growth media in 50 ml falcon tubes to create low and high supernatant dilutions as described previously. Finally, the ancestral, LB-exposed control and supernatant-exposed evolved populations were inoculated into these wells using a stainless-steel flame-sterilized cryoreplicator (~1 µl transfer volume, Boekel). Bacterial growth was recorded at 24-hr intervals for 3 days (OD 600 nm; Tecan Sunrise spectrophotometer) and tolerance were estimated as the growth reduction of evolved *R. solanacearum* strains in *Pseudomonas* supernatant relative to the ancestral strains.

Determining *R. solanacearum* tolerance to a subset of *Pseudomonas* secondary metabolites

In a previous study, we analysed and identified secondary metabolite clusters in *Pseudomonas* genomes using AntiSMASH. Key metabolites (DAPG, pyoluteorin, and orfamides A and B) were identified, shown to be produced and showed evidence of antimicrobial activity during in vitro experiments (Clough et al., 2022). To directly test if the supernatant-exposed *R. solanacearum* populations had evolved tolerance to identified candidate antimicrobials, we measured the growth of ancestral and evolved bacteria isolated from the low supernatant concentration in the presence of single compounds using chemical standards. Only the "low supernatant" selection environment was used to limit the workload due to a high number of treatments and because the level of tolerance evolved was relatively similar in the low compared to high supernatant treatment. The standards for orfamide A, orfamide B, pyoluteorin, and DAPG were purchased from Santa Cruz Biotechnology to test their efficacy against *R. solanacearum* strains. Stocks were made in 100% dimethyl sulfoxide, with the exception of DAPG, which was prepared in 100% methanol and stored at −20 °C. To investigate tolerance evolution to DAPG, ancestral strains, supernatant-exposed, and LB-exposed control populations were grown for 24 hr at 28 °C in 100% LB broth and then inoculated (1×10^6 CFU/ml) into LB broth at the following DAPG concentrations: 1,000, 500, 100, 50, and 0 µM (LB broth control). Bacterial densities were recorded as optical density at 0 and 24 hr and 48 and 72 hr after inoculation with a spectrophotometer (OD 600 nm). Due to the relatively high price of other synthetic antimicrobials, only a subset of the *R. solanacearum* strains (#1 and #7) were used to test tolerance evolution to pyoluteorin and orfamides A and B—with both strains tested for tolerance to pyoluteorin, and strain #1 only for the orfamides. As these metabolites were predicted to be produced by *Pseudomonas* CHA0 and Pf-5 strains, we only tested the evolved *R. solanacearum* populations, which had been exposed to the supernatants of these two *Pseudomonas* strains in low supernatant concentration. The selected *R. solanacearum* populations were revived and grown as described previously in the fitness assays. Their tolerance to 100 µM concentrations of pyoluteorin and each orfamide suspended in 100% LB broth was then measured. After inoculation, bacterial densities were recorded as optical density at 0 and 24 hr and 48 and 72 hr with a spectrophotometer (OD 600 nm).

Statistical analyses

All analyses were conducted in R (R Core Team, 2023). Multiple statistical approaches were used to analyse the datasets, including ANOVA, repeated measures ANOVA and Tukey contrasts. For all datasets which included a temporal structure (including the selection experiment and growth in specific *Pseudomonas* metabolites) repeated measures ANOVA between subject and random effects were used with the lme4 (1.1-27.1) and nlme (v3.1-163) R packages. Further pairwise comparisons were conducted in these models using Tukey contrasts with Bonferroni-corrected *p*-values. The selection experiment data were manipulated in Excel in an additional way by quantifying the supernatant inhibition by subtracting OD values observed in the supernatant treatments from the LB-control treatments and converting these into growth reductions shown as a percentage. This form of data manipulation was also carried out for the fitness assays and metabolite-exposed datasets. The percentage growth reduction datasets were also analysed through the repeated measured ANOVA analyses described above. Two-way ANOVA was also used to consider mean differences between treatments at the final time point of the selection experiment, and hence for all fitness assay data (which was shown as an average over the time points). Contrasts were further analysed using post hoc Tukey tests with 95% confidence levels. Tables of detailed analyses can be found in Supplementary Tables S4–S32. All statistical analyses and graphs were produced using R (R Foundation for Statistical Computing, R Studio Version.3.4.4). Packages included: ggplot (v3.4.3), dplyr (v1.1.2), magrittr (v2.0.3), multcomp (1.4-25), plyr (v1.8.8), RColorBrewer (v1.1-3), readr (v2.1.2), reshape2 (v1.4.4), and tidyr (v1.3.0). Colourblind-friendly palettes were used in all figures (viridis, magma, plasma, and inferno).

Results

Ralstonia solanacearum densities increased over time in the presence of *Pseudomonas* supernatants

We first explored the mean growth of all *R. solanacearum* strains in the presence of two supernatant concentrations during the selection experiment (Figure 1A and B). More detailed statistics results can be found in Supplementary Tables S4–S7. When averaged over all *Pseudomonas* strains, *R. solanacearum* growth was lower in both *Pseudomonas* supernatant concentrations relative to the control treatments ($F_{1, 488} = 142.752$, $p < 0.001$, Figure 1, Supplementary Table S4). Moreover, *R. solanacearum* densities were lower in the high supernatant compared to low supernatant concentration, indicative of relatively higher antimicrobial activity ($F_{1, 488} = 53.878$, $p < 0.001$, Figure 1A and B, Supplementary Table S4). For a clearer insight into supernatant effects, we next analysed the pathogen growth dynamics in the form of growth reduction by supernatants relative to the bacterial

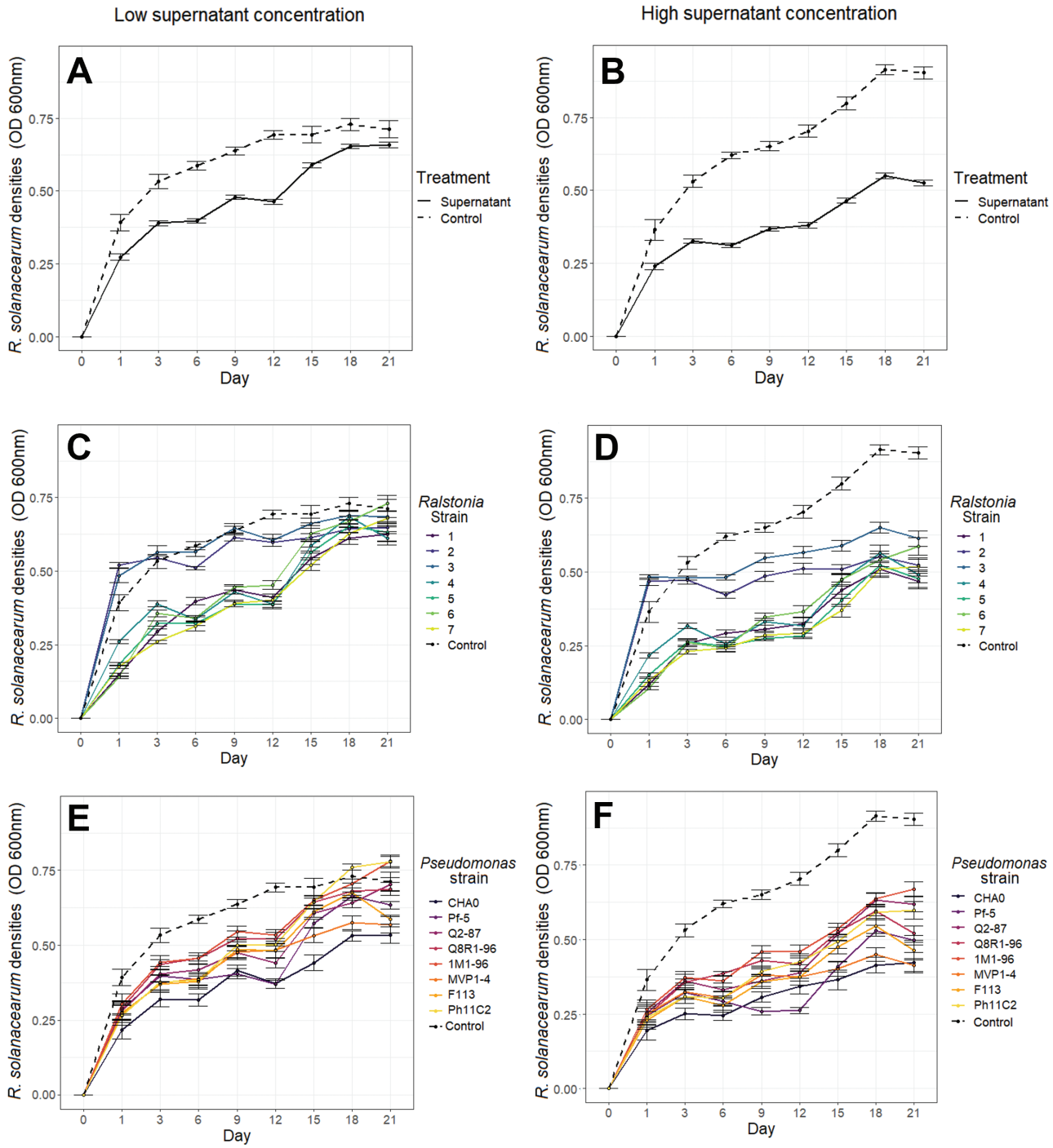

Figure 1. *R. solanacearum* population density dynamics in low (panels A, C, and E) and high (panels B, D, and F) *Pseudomonas* supernatant concentrations. Panels (C) and (D) show the effect of *R. solanacearum* strain variation (averaged over *Pseudomonas* strains), and panels (E) and (F) show the effect of *Pseudomonas* strain variation (averaged over *R. solanacearum* strains) on *R. solanacearum* densities in low (C and E) and high supernatant (D and F) concentrations. The dashed black line in each panel denotes *R. solanacearum* average densities in the absence of *Pseudomonas* supernatant (control). All bars show the standard error of the mean (±1 SEM). Statistics related to this results section can be found in Supplementary Tables S4–S7.

growth in the absence of supernatant (control treatment). A similar result was observed: *R. solanacearum* populations exposed to the high supernatant concentration experienced a greater reduction in their growth compared to the low supernatant concentration ($F_{1,\ 446} = 143.66$, $p < 0.001$, Figure 1A and B, Supplementary Table S5). The relative growth suppression by *Pseudomonas* supernatants was also reduced during the course of the experiment in both low ($F_{7,\ 1561} = 81.972$, $p < 0.001$, Figure 1A) and high ($F_{7,\ 1561} = 18.31$, $p < 0.001$, Figure 1B, Supplementary Table S5) supernatant concentrations. A reduction in suppression was especially clear in the low supernatant concentrations where pathogens reached similar densities as in the control treatment at the end of the selection experiment. An additional unexpected observation was the greater growth of *R. solanacearum* control populations in the high compared to low supernatant treatment even

though the growth media was diluted with sterile water more in this environment.

We next compared how the relative pathogen growth reduction was affected by *R. solanacearum* and *Pseudomonas* strain identities (Figure 1C and D). The growth reduction differed significantly between *Ralstonia* strains ($F_{6, 441}$ = 78.601, p < 0.001, Figure 1C and D, Supplementary Table S5). Post hoc analyses revealed that strains #2 and #3 were reduced to a lesser extent relative to the other five *R. solanacearum* strains (Supplementary Table S6). The growth reduction of *R. solanacearum* strains was not influenced by supernatant concentration ($F_{6, 434}$ = 0.693, p = 0.6554, Figure 1C and D, Supplementary Table S5). Similarly, the overall growth reduction by *Pseudomonas strains* varied significantly during the experiment ($F_{7, 440}$ = 8.95, p < 0.001, Figure 1E and F, Supplementary Table S5) with post hoc analyses revealing the strain CHA0 being more suppressive compared to Q2-87, Q81R-96, F113, and Ph11C2 strains (Supplementary Table S7). The mean suppression by *Pseudomonas* strains was not affected by the supernatant concentration ($F_{7, 432}$ = 0.3566, p = 0.9268, Figure 1E and F, Supplementary Table S5). Together these results suggest that pathogen growth suppression was relatively stronger in high supernatant concentration and depended on both *R. solanacearum* and *Pseudomonas* strain identities.

Ralstonia solanacearum strains evolved tolerance to *Pseudomonas* antimicrobials

Fitness assays were conducted after the selection experiment to compare the growth of ancestral, LB-exposed (control), and supernatant-exposed *R. solanacearum* populations in LB broth and in the presence of ancestral *Pseudomonas* supernatants. More detailed statistics results can be found in Supplementary Tables S8–S10. It was found that the densities of all *R. solanacearum* populations were significantly reduced in the presence of *Pseudomonas* supernatants as opposed to the LB broth control treatment ($F_{1, 4478}$ = 700.3, p < 0.001, Figure 2A and B, Supplementary Table S8).

We then explored if the prior exposure to supernatants affected *R. solanacearum* growth by comparing the percentage growth reduction by supernatants relative to *R. solanacearum* growth in LB media without supernatants. The growth of all *R. solanacearum* populations was reduced irrespective of their evolutionary history when exposed to *Pseudomonas* supernatants ($F_{2, 2237}$ = 36.44, p < 2e-16, Figure 2A and B, Supplementary Table S8). However, the growth of ancestral *R. solanacearum* populations was reduced the most, followed by LB-exposed control and supernatant-exposed *R. solanacearum* populations, which were reduced the least. Post hoc analyses showed the reduction of the LB-exposed and ancestral *R. solanacearum* populations was not significantly different (p = 0.252), but both ancestral (Tukey: p < 0.0001) and LB-exposed control (Tukey: p < 0.0001) populations differed significantly from the supernatant-exposed *R. solanacearum* populations (Supplementary Table S9). Overall, the reduction in *R. solanacearum* growth was greater in the high *Pseudomonas* supernatant concentration (*Supernatant concentration*: $F_{1, 2238}$ = 16.53, p < 0.0001, Figure 2B, Supplementary Table S8), which is in line with the population density dynamics observed during the selection experiment. However, this effect was driven by ancestral strains whose growth was most clearly affected by *Pseudomonas* supernatant concentration (*Supernatant concentration*Evolutionary history*: $F_{2, 2234}$ = 7.015, p < 0.001, Figure 2B, Supplementary Table S8), whereas post hoc analyses showed concentration had no effect on the growth of LB-exposed control or supernatant-exposed evolved populations (p > 0.05, Supplementary Table S10). This indicates that *R. solanacearum* populations evolved increased tolerance to *Pseudomonas* supernatants during the selection experiment and that this effect was similar in both supernatant concentrations.

The effect of *R. solanacearum* strain identity for the evolution of tolerance

We next explored the role of *R. solanacearum* strain identity on tolerance evolution against *Pseudomonas* supernatants in both supernatant concentrations (Figure 3). More detailed

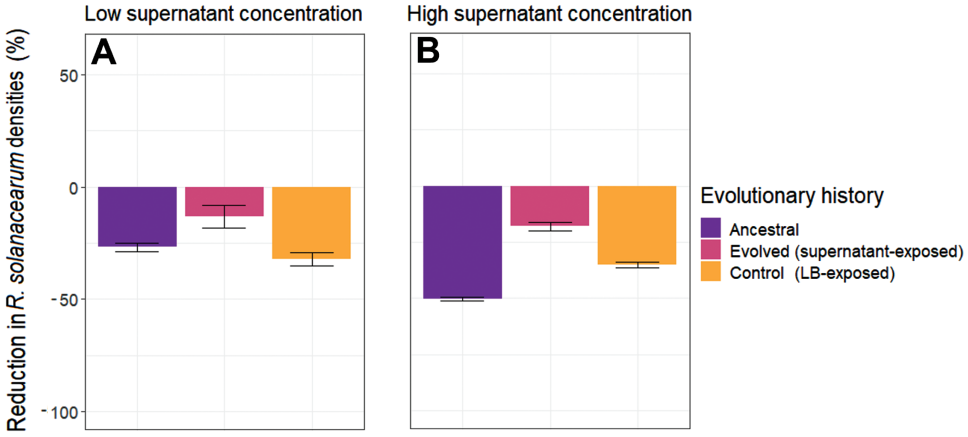

Figure 2. Evolution of tolerance in terms of pathogen growth reduction in the presence of *Pseudomonas* supernatants compared to supernatant-free growth media. Tolerance was measured only in the same supernatant concentration *R. solanacearum* strains had evolved during the selection experiment and only against the *Pseudomonas* strain they had previously been exposed to. Bar plots show the percentage reduction in density of ancestral (purple), evolved (pink), and control (orange) *R. solanacearum* selection lines when grown in *Pseudomonas* supernatant compared to the control treatment with standard error of the mean (±1 SEM). Panels (A) and (B) show populations exposed to low and high supernatants, respectively. All data were averaged over *R. solanacearum* and *Pseudomonas* strains across two measurement time points during the fitness assays (24 and 72 hr). Statistics related to this results section can be found in Supplementary Tables S8–S10.

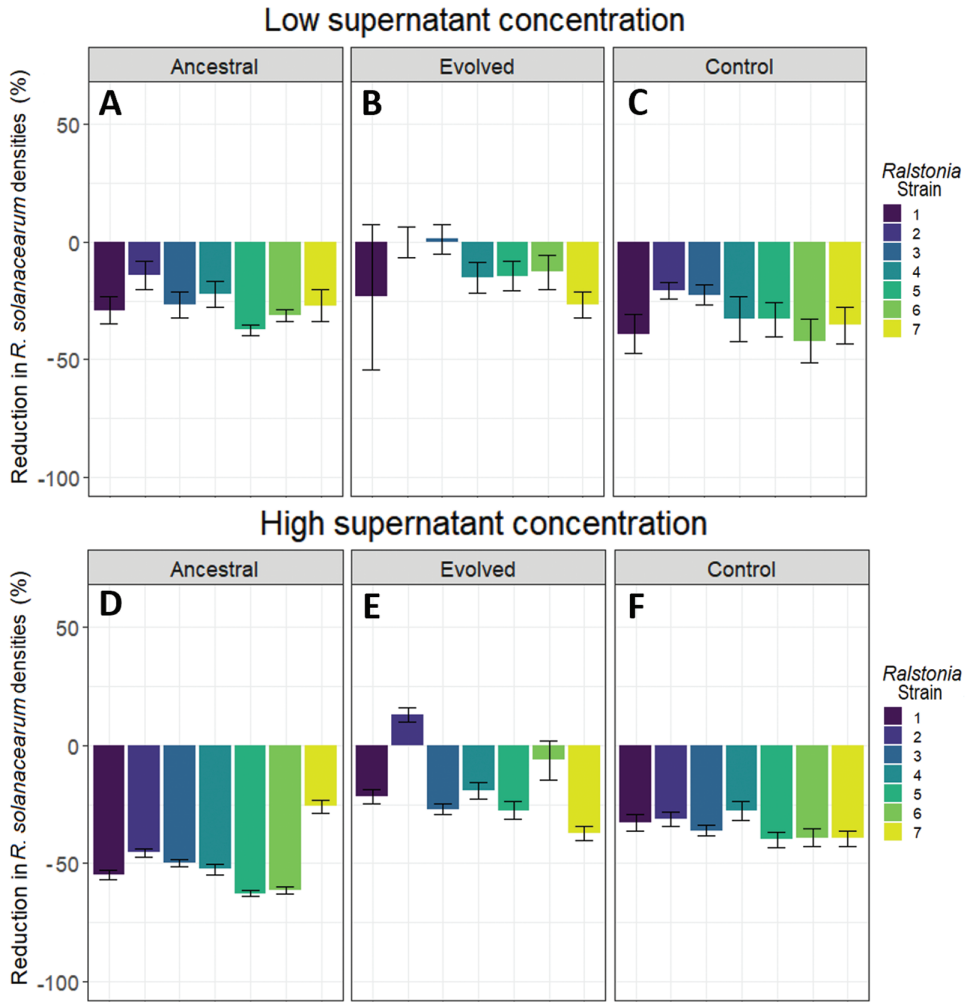

Figure 3. The significance of *R. solanacearum* strain identity for the evolution of tolerance. Tolerance was measured as pathogen growth reduction in the presence of *Pseudomonas* supernatants compared to supernatant-free growth media. Only the same supernatant concentration and *Pseudomonas* strains *R. solanacearum* strains had been exposed to during the selection experiment were used for the analysis. Bar plots show the percentage reduction in density of ancestral (A and D), supernatant-exposed evolved (B and E), and LB-exposed control (C and F) *R. solanacearum* selection lines when grown in *Pseudomonas* supernatant compared to the control treatment; with standard error of the mean (±1 SEM). Panels (A–C) and (D–F) show populations exposed to low and high supernatants, respectively. All data were averaged over *Pseudomonas* strains across two measurement time points during the fitness assays (24 and 72 hr). Statistics related to this results section can be found in Supplementary Tables S11–S13.

statistics results can be found in Supplementary Tables S11–S13. The growth of all ancestral *Ralstonia* strains was reduced in the presence of *Pseudomonas* supernatants in general, but this effect varied between strains (*identity effect for Ralstonia*: $F_{6,\ 2233} = 4.502$, $p = 0.000153$, Figure 3, Supplementary Table S11). To explore this in more detail, we compared the responses of ancestral *Ralstonia* strains independently in low and high supernatant concentrations and found that the pathogen growth reduction varied between the strains in the high *Pseudomonas* supernatant concentration ($F_{6,329} = 39.41$, $p < 0.001$, Figure 3D, Supplementary Table S11). Post hoc analyses showed that the densities of strains #5 and #6 were reduced the most, while supernatants had the least effect on strain #7 (Supplementary Table S12). In contrast, no variation was observed amongst ancestral strains grown when exposed to the low supernatant concentration ($F_{6,329} = 1.938$, $p = 0.0742$, Figure 3A, Supplementary Table S11). In the case of LB-exposed control populations, no *R. solanacearum* strain variation was observed in low ($F_{6,329} = 1.14$, $p = 0.338$, Figure 3C, Supplementary Table S11) or high ($F_{6,329} = 2.029$, $p = 0.0614$, Figure 3F, Supplementary Table S11) supernatant concentrations and all strains showed similar levels of tolerance as the ancestral populations. In contrast, supernatant-exposed evolved populations showed strain-specific tolerance evolution ($F_{6,889} = 3.105$, $p = 0.00514$, Supplementary Figure S1B + E). While no clear differences were found in the low supernatant concentration (*identity effect for evolved Ralstonia:* $F_{6,441} = 0.651$, $p = 0.689$, Figure 3B, Supplementary Table S11), strains #2 and #6 showed increased levels of tolerance in the high supernatant concentration ($F_{6,441} = 15.36$, $p < 0.0001$, Figure 3E, Supplementary Table S13). Together, these results suggest that *R. solanacearum* strains varied in their innate tolerance and tolerance evolution mainly at high supernatant concentration treatment.

The effect of *Pseudomonas* strain identity for the evolution of tolerance

We next analysed if *R. solanacearum* tolerance evolution was affected by which *Pseudomonas* strain they had been exposed to during the selection experiment (Figure 4). More

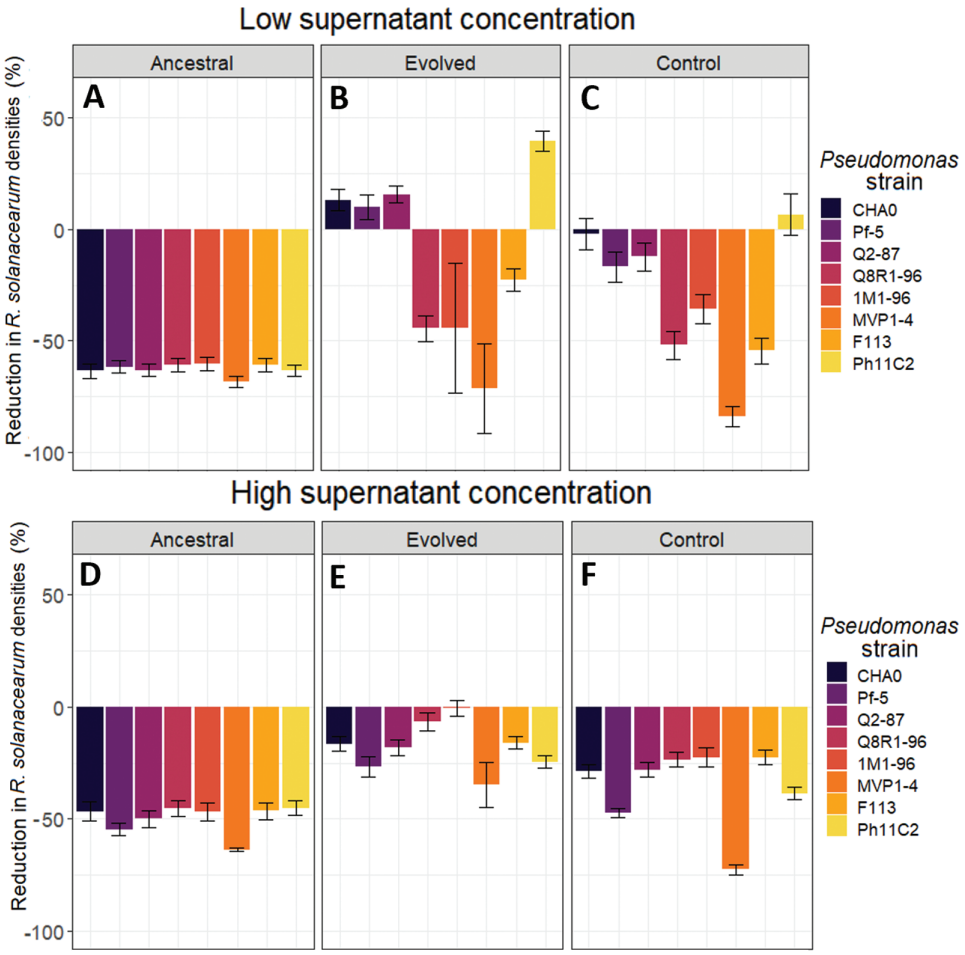

Figure 4. The significance of *Pseudomonas* strain identity for the evolution of tolerance. Tolerance was measured as pathogen growth reduction in the presence of *Pseudomonas* supernatants compared to supernatant-free growth media. Only the same supernatant concentration of *Pseudomonas* strains which *R. solanacearum* strains had been exposed to during the selection experiment were used. Bar plots show the percentage reduction in density of ancestral (A and D), evolved (B and E), and control (C and F) *R. solanacearum* selection lines when grown in *Pseudomonas* supernatant compared to the control treatment with standard error of the mean (±1 SEM). Panels (A–C) and (D–F) show populations exposed to low and high supernatants, respectively. All data were averaged over *R. solanacearum* strains across two measurement time points during the fitness assays (24 and 72 hr). Statistics related to this results section can be found in Supplementary Tables S14–S19.

detailed statistics results can be found in Supplementary Tables S14–S19. While all *Pseudomonas* strains were equally effective at suppressing ancestral *R. solanacearum* strains in low supernatant concentration ($F_{7, 328} = 0.81$, $p = 0.58$, Figure 4A, Supplementary Table S14), *Pseudomonas* strain MVP1-4 clearly showed higher growth reduction relative to other *Pseudomonas* strains in the high supernatant concentration ($F_{7, 328} = 3.424$, $p = 0.00151$, Figure 4D, Supplementary Tables S14 and S15). In the case of LB-exposed control populations, differences between *Pseudomonas* strains were observed already in the low supernatant concentration ($F_{7, 328} = 21.31$, $p < 0.0001$, Figure 4C, Supplementary Table S14), with post hoc analyses revealing a clear increase in tolerance to Ph11C2 and CHA0 strains, while *R. solanacearum* strains remained highly susceptible to MVP1-4, F113, and Q8R1-96 strains (Supplementary Table S16). This was also the case in the high supernatant concentration ($F_{7, 328} = 30.97$, $p < 0.001$, Figure 4F, Supplementary Table S14), with post hoc analyses highlighting the relatively high susceptibility of *R. solanacearum* strains to MVP1-4, Pf-5, and Ph11C2 strains and less clear tolerance evolution to Ph11C2 and CHA0 strains (Supplementary Table S17). Similarly, supernatant-exposed evolved populations showed high variability in their tolerance to different *Pseudomonas* strains, and interestingly, this variation was greater at low supernatant concentration evolved populations (*supernatant concentration*Pseudomonas:* $F_{7, 440} = 8.393$, $p < 0.001$, Figure 4B, Supplementary Table S14). Specifically, *R. solanacearum* evolved increased tolerance to Ph11C2, Q2-87, CHA0, and Pf-5 strains, but remained susceptible to MVP1-4, 1M1-96, and Q8R1-96 strains in low supernatant concentration ($F_{7, 440} = 8.393$, $p < 0.001$, Figure 4B, Supplementary Table S18). Qualitatively similar patterns were observed in high supernatant concentration ($F_{7, 440} = 5.181$, $p < 0.001$, Figure 4E, Supplementary Table S19), but the level of tolerance evolution was generally lower. Together, these results show that *R. solanacearum* tolerance evolution was generally weakest against MVP1-4 strain and strongest against Ph11C2, CHA0, Pf-5, and Q2-87 strains of *Pseudomonas*.

Tolerance evolution was linked to *R. solanacearum* ability to grow in the presence of DAPG, Orfamides A and B, and Pyoluteorin antimicrobials produced by *Pseudomonas*

Based on our previous work on this system, we tested the *R. solanacearum* tolerance to four candidate compounds that were found in *Pseudomonas* genomes and were secreted

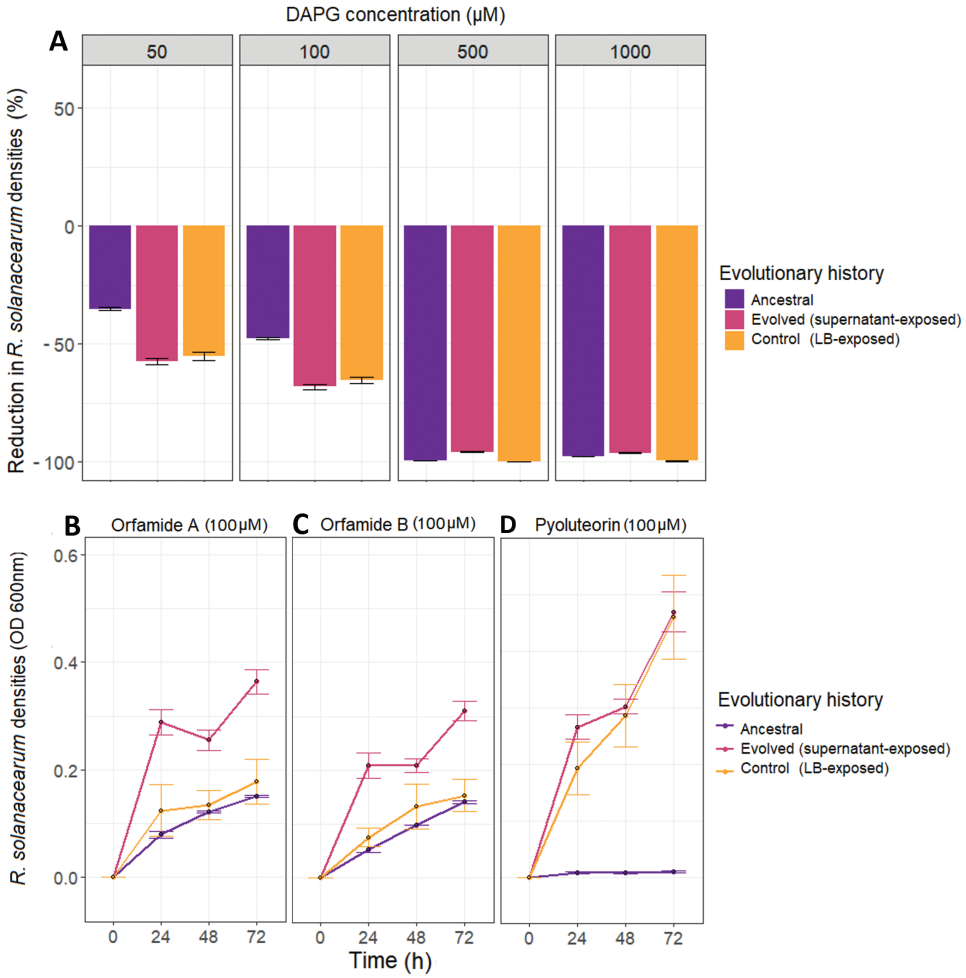

Figure 5. Evolution of tolerance in (A) terms of pathogen growth reduction in the presence of DAPG compared to DAPG-free growth medium (LB) and in terms of comparing the growth of ancestral LB-exposed control and supernatant-exposed evolved *R. solanacearum* populations in 100 μM of orfamide A (B), orfamide B (C), and pyoluteorin concentrations (D). (A) Bar plots show the percentage reduction in density of ancestral (purple), evolved (pink), and control (orange) *R. solanacearum* selection lines when grown in four DAPG concentrations 1,000, 500, 100, and 50 μM compared to the control treatment grown in the absence of DAPG with standard error of the mean (±1 SEM). All data were averaged over *R. solanacearum* and *Pseudomonas* strains across two measurement time points during the fitness assays (24 and 72 hr). (B–D) The line graphs show the ability of ancestral (purple line), evolved (pink line), and control (orange line) *R. solanacearum* selection lines to grow in the presence of selected metabolites over time. The evolved selection lines in each panel are averaged over the two *Pseudomonas* strains (CHA0 and Pf-5), and in panel (D), each line is additionally averaged over two *R. solanacearum* strains (#1 and #7). Statistics related to this results section can be found in Supplementary Tables S20–S32.

to the environment, based on metabolomics (Clough et al., 2022). These compounds included DAPG, orfamides A and B, and pyoluteorin, which were available as chemical standards. Only the evolved populations originating from the low supernatant concentration were used in these analyses, and while the effects of DAPG were tested with all *R. solanacearum* strains in multiple concentrations, only a subset of *Ralstonia* strains were used to analyse the effects of other candidate compounds due to the high price of chemical standards.

Tolerance to DAPG was explored by averaging data over *R. solanacearum* and *Pseudomonas* strains and comparing the percentage growth reduction by DAPG relative to *R. solanacearum* growth in LB media (more detailed growth trajectories for each *Ralstonia* strain over time can be found in Supplementary Figures S1 and S2). More detailed statistics results can be found in Supplementary Tables S20–S26. It was found that DAPG suppressed all *R. solanacearum* strains in a concentration-dependent manner, with the largest growth reduction observed in the two highest DAPG concentrations that were used: 1,000 and 500 μM ($F_{3, 4644} = 2234$, $p < 0.001$, Figure 5A, Supplementary Table S20). The growth reduction was also slightly affected by the evolutionary history and this effect varied with DAPG concentration (*Evolutionary history*Concentration*: $F_{6, 4636} = 71.96$, $p < 0.001$, Figure 5A, Supplementary Table S20). Interestingly, in the two lowest DAPG concentrations (50 and 100 μM), the growth of both LB-exposed control and supernatant-exposed evolved populations were reduced relatively more compared to ancestral populations, which indicates that these populations suffered some form of cost at low DAPG concentrations (*Evolutionary history in 50 μM DAPG*: $F_{2, 1117} = 80.33$, $p < 0.001$, Figure 5A; *Evolutionary history in 100 μM DAPG:* $F_{2, 1117} = 95.83$, $p < 0.0001$, Figure 5A, Supplementary Tables S20–S22). While no difference between LB-exposed control and supernatant-exposed evolved populations was found at 50 μM ($p = 0.718$) and 100 μM ($p = 0.38$) DAPG concentrations, the supernatant-exposed evolved populations were slightly less reduced than the LB-exposed control population ($p = 0.024$) at the highest 500 μM DAPG concentration; however, this result did not have a great impact on

the growth trajectories of the populations (Supplementary Table S23). While the ancestral *R. solanacearum* strains did not differ in their innate tolerance to DAPG ($F_{6, 1506} = 1.598$, $p = 0.144$, Supplementary Figure S1), the growth reduction of LB-exposed *R. solanacearum* strains #6 and #4 showed the highest susceptibility ($F_{6, 1337} = 9.98$, $p < 0.001$, Supplementary Figure S2A, Supplementary Tables S20 and S24). In the case of supernatant-exposed evolved *R. solanacearum* strains, more variation was found ($F_{6, 1785} = 12.22$, $p < 0.001$, Supplementary Figure S2A, Supplementary Tables S20 and S25). Specifically, the growth of strains #2, #3, and #7 was most reduced by DAPG (unable to grow to the highest densities), while strains #1, #4, #5, and #6 were the least susceptible to DAPG. The *Pseudomonas* supernatant species identity also influenced *Ralstonia* growth reduction with those populations that had been exposed to MVP1-4 experiencing the smallest growth reduction (*strain identity of Pseudomonas strain*: $F_{7, 1784} = 2.409$, $p < 0.001$, Appendix, Figure 2B, Supplementary Tables S20 and S26).

Only a subset of *R. solanacearum* strains were used for analysing the inhibitory effects of orfamides and pyoluteorin. More detailed statistics results can be found in Supplementary Tables S27–S32. In the case of orfamide tolerance, only the *R. solanacearum* strain #1, which had been exposed to LB or *Pseudomonas* CHA0 and Pf-5 supernatants in the selection experiment was used. As the effect of *Pseudomonas* strain identity was nonsignificant ($F_{1, 32} = 0.261$, $p = 0.6128$, Figure 5B and C, Supplementary Table S27), growth data were pooled over both *Pseudomonas* strains. While the growth of *R. solanacearum* did not vary between orfamides A and B ($F_{1, 32} = 1.72$, $p = 0.199$, Figure 5B and C, Supplementary Table S27), by the final time point the supernatant-exposed evolved populations reached relatively higher densities compared to LB-exposed control ($p < 0.001$) or ancestral ($p < 0.001$) populations when grown in 100 µM orfamide concentrations ($F_{2, 31} = 60.67$, $p < 0.001$ Figure 5B and C, Supplementary Tables S28 and S29). Moreover, no difference in ancestral and LB-exposed control populations was observed ($p = 0.698$), which suggests that tolerance of control populations was unlikely driven by orfamide tolerance. In the case of pyoluteorin, *R. solanacearum* strains #1 and #7 that had been exposed to *Pseudomonas* CHA0 and Pf-5 supernatants during the selection experiment were used. Due to nonsignificant effect of *Pseudomonas* strain identity ($F_{1, 32} = 0.0211$, $p = 0.8854$, Figure 5D, Supplementary Table S30), growth data were again pooled over the two strains. While the growth of *R. solanacearum* did not vary between strains #1 and #7 ($F_{1, 32} = 0.274$, $p = 0.6014$, Figure 5C, Supplementary Table S30), both LB-exposed control and supernatant-exposed evolved populations reached relatively higher densities than the ancestral strains ($F_{2, 31} = 178.2$, $p < 2e\text{-}16$ Figure 5D, Supplementary Table S31). As no difference was found between LB-exposed control and supernatant-exposed evolved populations ($p > 0.05$, Supplementary Table S32), it is possible that spontaneous pyoluteorin tolerance could partly explain the improved tolerance of control selection lines to *Pseudomonas* supernatants.

Discussion

Building on previous work demonstrating the ability of *R. solanacearum* to evolve tolerance to plant antimicrobials (Alderley et al., 2022; Li et al., 2021), the aims of this study were to investigate if *R. solanacearum* was able to develop tolerance to antimicrobials produced by *Pseudomonas* biocontrol bacterial agents, and if this was dependent on the *R. solanacearum* and *Pseudomonas* strain identities. We found that all *Pseudomonas* strains were initially able to suppress pathogen growth, with some strains causing greater suppression than others. Comparisons between ancestral, LB-exposed control, and supernatant-exposed evolved populations after the selection experiment revealed potential pathogen tolerance *to Pseudomonas* antimicrobials, which were likely driven by correlated responses in control treatments due to adaptation to the growth media. While tolerance evolution was little affected by the *R. solanacearum* strain identity, it varied more depending on the *Pseudomonas* strain identity, with MVP1-4 strain showing consistent growth suppression with no clear evidence for tolerance evolution. Based on previous study, we tested the inhibitory potential of a few *Pseudomonas* antimicrobials against ancestral, LB-exposed control, and supernatant-exposed *R. solanacearum* strains to see if we could disentangle tolerance to the supernatant antimicrobials versus growth media adaptation. We found that tolerance was linked to improved growth in the presence of most of the tested compounds, with the clearest effects seen in the presence of orfamides, which were previously found to be produced in these experimental growth conditions (Clough et al., 2022). Together these results show that *R. solanacearum* tolerance evolution could potentially reduce the long-term efficiency of *Pseudomonas*-based biocontrol. However, these results need to be validated in more natural environments in the future to test if tolerance could evolve in a soil or plant rhizosphere.

Our results showed that all eight of our *Pseudomonas* strains had the ability to suppress all seven *R. solanacearum* isolates. These effects were sublethal and constrained *R. solanacearum* growth in a concentration-dependent manner. Similar results have been previously reported by Raza et al. (2016), who conducted agar and soil assays with one *Pseudomonas* strain and one *R. solanacearum* strain pair revealing similar concentration-dependent suppressive effects of antimicrobial volatile organic compounds. The strain CHA0 was the most suppressive strain against *R. solanacearum* growth, which is in line with previous research where it has been found to be a highly effective *Pseudomonas* plant growth-promoting bacteria (Haas & Keel, 2003; Hu et al., 2016; Jousset et al., 2006). Interestingly, strain MVP1-4 was also a highly effective strain. More research is hence required to find out if MVP1-4 is able to produce any unique antimicrobials or whether it is simply able to produce higher amounts of different antimicrobials relative to other *Pseudomonas* strains, which could be confirmed using metabolomics (Yasmin et al., 2017). In contrast, CHA0 and Pf-5 are very well-studied *Pseudomonas* strains, which could also explain their relatively largest metabolic repertoire reported (Gabriel et al., 2006; Haas & Keel, 2003). Comparative genomics focusing on secondary metabolism could perhaps reveal more similarities between MVP1-4 and similarly suppressive CHA0 and Pf-5 strains. Some *Pseudomonas* metabolites such as DAPG are universally produced by these bacterial species (Haas & Défago, 2005), which could explain why all the *Pseudomonas* strains used in this study showed pathogen suppression. In contrast, the high suppressiveness of certain *Pseudomonas* strains was likely linked to unique secondary metabolite clusters, such as orfamides and pyoluteorin identified in the genome of strain

CHA0 (Clough et al., 2022; Ma et al., 2016). It is also possible that *Pseudomonas* secondary metabolism and antimicrobial activity are altered when directly interacting with the pathogen, which we would have missed in our setting that exposed the pathogen directly to filtrated supernatants. Furthermore, *Pseudomonas* could also use other inhibitory mechanisms to reduce *R. solanacearum* growth when in direct interaction as has been found with several other bacteria (Granato et al., 2019). Small variation in susceptibility was also observed between *R. solanacearum* strains during the selection experiment. For example, *R. solanacearum* strain #6 grew to the highest densities by the end of the selection experiment, which suggests that it had the greatest innate tolerance to the *Pseudomonas* antimicrobials—a result, which was also confirmed in separate fitness assays. It was also found that *R. solanacearum* strains isolated from the same geographical area (e.g., strains #2 and #3) followed the same growth dynamics, which could result from potential close genetic relatedness or prior local adaptation. While all *R. solanacearum* strains used in this study belong to the same clonal Phylotype IIB sequevar 1 formerly known as race3 biovar2 strain (Clarke et al., 2015), recent data show that they differ phenotypically (Farnham, 2022) and can evolve rapidly at least in the lab (Alderley et al., 2022). In the future, it would be interesting to study what caused the divergent density trajectory of *R. solanacearum* strains #2 and #3. One explanation could be mutations in the phcA regulatory locus, which can sporadically emerge during storage or when culturing in lab medium (Khokhani et al., 2017; Peyraud et al., 2016), resulting in improved bacterial growth. While sequencing is needed to confirm this in the future no such genetic changes were observed in a previous study where one of the *R. solanacearum* strains used in this experiment (#1) was cultured in Casamino acid-Peptone-Glucose (CPG) medium. However, as we used LB medium in this experiment the outcome might have been different. Moreover, as all the *R. solanacearum* strains we used were genetically highly similar, it will be important to validate our tolerance evolution results with a genetically more diverse set of strains to generalize our findings across the Ralstonia solanacearum species complex. *Pseudomonas* strains tested included different species (*P. protegens* and *Pseudomonas fluorescens* species), which likely explains why relatively more variation was observed between them, which could also be true for the evolutionary trajectory of different *Ralstonia* species.

We found that the ancestral and LB-exposed control *R. solanacearum* populations were more susceptible to *Pseudomonas* antimicrobials compared to the supernatant-exposed evolved *R. solanacearum* strains at the end of the selection experiment. This indicates that continued antimicrobial exposure can select for *R. solanacearum* tolerance to *Pseudomonas* antimicrobials in just a few weeks in laboratory conditions. As no extinctions were observed in any *R. solanacearum* populations over the selection experiment, our results suggest that *Pseudomonas* supernatants had sublethal effects, or alternatively, consistent production by directly interacting bacteria would have been required for more effective killing (Fravel, 1988). Tolerance evolution was most clear with supernatant-exposed evolved *R. solanacearum* populations that reached considerably higher densities than both the LB-exposed control and ancestral *R. solanacearum* populations when re-exposed to supernatants in the fitness assays. Similar effects were also observed in direct metabolite assays with the subset of *R. solanacearum* strains. In the case of DAPG, only marginal evidence of tolerance at a concentration of 500 μM was found, where the growth of supernatant-exposed evolved population was slightly less suppressed by the supernatants compared to ancestral and LB-exposed populations. It is hence unlikely that adaptation to DAPG would explain the loss of suppression by the supernatants. Moreover, both LB-exposed and evolved supernatant-exposed populations grew worse than ancestral strains in 50 and 100 μM DAPG concentrations—indicative of reduced tolerance to DAPG. One explanation for these results is that no DAPG was produced by the *Pseudomonas* strains in these culture conditions (Clough et al., 2022), which would explain why no tolerance to DAPG was selected. Alternatively, DAPG tolerance could have been traded-off with the tolerance to other compounds, potentially due to antagonistic pleiotropy.

In a previous study, using liquid chromatography–mass spectrometry we showed orfamide A could be detected in the supernatant of CHA0 under the same growth conditions used in this selection experiment (Clough et al., 2022). In line with this, the supernatant-exposed evolved populations had the greatest tolerance to orfamides A and B compared to the ancestral and LB-exposed control populations. This result suggests that orfamide tolerance could partly explain the evolution of improved tolerance observed in our selection experiment. Orfamides A and B are known to be associated with the same operon, which suggests that they are co-expressed and could hence explain observed tolerance to both compounds (Ma et al., 2016). While sequencing studies are needed to unravel the potential molecular mechanism of improved *R. solanacearum* orfamide tolerance, one possibility could be the use of multidrug efflux pumps, which *R. solanacearum* uses to protect itself against plant defences (Brown et al., 2007). Moreover, as *R. solanacearum* is a soil-borne pathogen, it is highly innately resistant to certain antibiotics produced by other bacteria, such as polymyxin B, which is an antimicrobial peptide produced by *Paenibacillus polymyxa* soil bacterium (Elphinstone et al., 1996). As orfamides are also lipopeptides, it is possible that potentially small genetic changes could have led to improved tolerance to other antimicrobial compounds.

In the pyoluteorin fitness assays, no difference was observed between the LB-exposed control and supernatant-exposed evolved populations, even though they both were clearly more tolerant to pyoluteorin compared to the ancestral strains. This suggests that this adaptation could have been driven indirectly by media adaptation or some other unknown factors unrelated to *Pseudomonas* supernatant selection. Pyoluteorin is a chlorinated polyketide antibiotic and its production is regulated by the DAPG precursor, monoacetylphloroglucinol (Kidarsa et al., 2011). The closely regulated biosynthetic gene clusters of DAPG and pyoluteorin are thus thought to have a close evolutionary history (Kidarsa et al., 2011). While evolution of antimicrobial tolerance via metabolic adaptations has been observed in other recent studies (Crabbé et al., 2019; Lopatkin et al., 2021) our results are difficult to explain without further detailed experiments and a better understanding of the link between antimicrobial tolerance and primary (growth) metabolism, which could be achieved for example via transcriptomics studies. Genome resequencing could also reveal potential mutations and mechanisms underlying orfamide and pyoluteorin tolerance and DAPG susceptibility. Surprisingly, some LB-exposed control populations showed increased levels of tolerance compared to

ancestral strains despite having never been exposed to antimicrobials during the selection experiment. One explanation for this is that the negative effects of antimicrobials were potentially compensated by the LB-exposed pathogen adapting to grow better in our experimental conditions (LB broth). It is also possible that high sodium chloride concentration present in the LB media could have indirectly selected for generalized stress tolerance, which correlated with tolerance to pyoluteorin. Such generalized stress tolerance adaptation could have been achieved, for example, via changes in efflux pump activity. Lower total concentration of sodium chloride in "high supernatant concentration" control treatment (diluted more with water) could have also affected media adaptation in this treatment during the selection experiment. In support of these ideas, it has previously been shown that *R. solanacearum* can adapt and diversify rapidly when grown in the lab media (Riley et al., 2001). Moreover, a recent study showed that *R. solanacearum* adaptation to the CPG growth media (without added sodium chloride) can lead to improved tolerance to antimicrobial isothiocyanate plant allelochemical secreted by *Brassica* plants (Alderley et al., 2022) with one of the strains that was also used in this study (#1—YO352). These findings are in line with previous studies demonstrating that antibiotic tolerance can evolve even in the absence of antibiotics due to media adaptation and associated pleiotropic effects (Knöppel et al., 2017). An additional experiment to determine adaptations to the growth medium could be conducted in the future by quantifying the fitness of evolved bacteria in "naïve" media, such as CPG, to differentiate between growth medium and metabolite-mediated adaptations.

Investigating the potential of a pathogen to evolve tolerance to a biocontrol agent is important for increasing the long-term efficacy of pathogen biocontrol. An optimal biocontrol agent would be one that exerts a broad range of efficacy against different, potentially co-occurring pathogen species and multiple different genotypes within each pathogen species. Furthermore, there is a clear need to identify biocontrol agents that do not drive strong resistance evolution on pathogens. While it might be difficult to identify generalist *Pseudomonas* strains that suppress multiple strains of pathogens over a long period of time, the antimicrobial activity range of biocontrol applications could be broadened by using combinations of biocontrol agents that show varying efficacy against different pathogen species and genotypes as demonstrated by Hu et al. (2016). Additionally, high biocontrol agent diversity could potentially aid the *Pseudomonas* to establish and colonize the rhizosphere, for example, via elevated siderophore production and improved iron acquisition (Hu et al., 2016). A recent study also demonstrated that co-occurrence of two *Pseudomonas* species can facilitate pyoluteorin production by another strain, suggesting that antimicrobial production by *Pseudomonas* could be facilitated in more diverse communities (Hansen et al., 2022). One novel future area of research would be to investigate the combinatory effects on an evolutionary timescale. For example, it has previously been shown that phage and bacterial biocontrol agents can be more effective in combination compared to when applied alone due to evolutionary trade-offs that can constrain the emergence of generalist resistance evolution (Wang et al., 2017). For example, using the strain MVP1-4 in combination with CHA0 *Pseudomonas* strain might be an effective way to constrain *R. solanacearum* growth over longer time periods as overall only very weak tolerance evolution against MVP1-4 was observed. Moreover, *Pseudomonas* species could also be combined with *R. solanacearum*-specific phages or highly suppressive *Bacillus* bacteria to improve their efficacy and biocontrol efficiency range (Wang et al., 2017). Lastly, it is not clear if similar evolutionary outcomes would take place in more natural environments such as in the water, soil or the plant rhizosphere where *R. solanacearum* is exposed also to other stresses (Álvarez et al., 2022; Hayes et al., 2022). Similarly, it is important to determine if *Pseudomonas* can produce antimicrobials under these growth conditions. Studying the tolerance evolution and strength of selection in the natural environment is hence required to develop a more realistic understanding of the role of rapid evolution for biocontrol outcomes. Some research has already started to take these next steps. For example, Landa et al. (2002) investigated the long-term colonization ability and secondary metabolism production of various *Pseudomonas* strains in the pea plant rhizosphere over 8 months. They noted clear differences in long-term colonization success between biocontrol strains, which could have resulted from evolutionary changes. Moreover, *R. solanacearum* has previously been shown to evolve in response to selection by bacteriophages or antibiotics produced by *B*acillus *amyloliquefaciens* bacterium in the tomato rhizosphere (Wang et al., 2017, 2019). Considering long-term effects in vivo, as well as in vitro, are therefore crucial steps that need to be taken to understand whether the antimicrobials-based biocontrol can be a successful strategy for long-term pathogen suppression.

Supplementary material

Supplementary material is available at *Journal of Evolutionary Biology* online.

Data availability

The data underlying this article are available in the Dryad Digital Repository at https://doi.org/10.5061/dryad.1g1jwsv3g

Author contributions

Sophie Clough (Conceptualization [equal], Data curation [equal], Formal analysis [equal], Investigation [equal], Methodology [equal], Validation [equal], Visualization [equal], Writing—original draft [equal], Writing—review & editing [equal]), John Elphinstone (Investigation [equal], Supervision [equal], Writing—review & editing [equal]), and Ville-Petri Friman (Conceptualization [equal], Formal analysis [equal], Funding acquisition [equal], Investigation [equal], Project administration [equal], Supervision [equal], Writing—review & editing [equal])

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Conflicts of interest

None declared.

References

Alderley, C. L., Greenrod, S. T. E., & Friman, V. P. (2022). Plant pathogenic bacterium can rapidly evolve tolerance to an antimicrobial plant allelochemical. *Evolutionary Applications*, *15*(5), 735–750. https://doi.org/10.1111/eva.13363

Álvarez, B., López, M. M., & Biosca, E. G. (2022). *Ralstonia solanacearum* facing spread-determining climatic temperatures, sustained starvation, and naturally induced resuscitation of viable but non-culturable cells in environmental water. *Microorganisms*, *10*(12), 2503. https://doi.org/10.3390/microorganisms10122503

Balaban, N. Q., Helaine, S., Lewis, K., … Zinkernagel, A. (2019). Definitions and guidelines for research on antibiotic persistence. *Nature Reviews Microbiology*, *17*(7), 441–448. https://doi.org/10.1038/s41579-019-0196-3

Becker, J., Eisenhauer, N., Scheu, S., & Jousset, A. (2012). Increasing antagonistic interactions cause bacterial communities to collapse at high diversity. *Ecology Letters*, *15*(5), 468–474. https://doi.org/10.1111/j.1461-0248.2012.01759.x

Brown, D. G., Swanson, J. K., & Allen, C. (2007). Two host-induced *Ralstonia solanacearum* genes, acrA and dinF, encode multidrug efflux pumps and contribute to bacterial wilt virulence. *Applied and Environmental Microbiology*, *73*(9), 2777–2786. https://doi.org/10.1128/AEM.00984-06

Chen, J., Yu, Y., Li, S., & Ding, W. (2016). Resveratrol and coumarin: Novel agricultural antibacterial agent against *Ralstonia solanacearum* in vitro and in vivo. *Molecules*, *21*(11), 1501. https://doi.org/10.3390/molecules21111501

Clarke, C. R., Studholme, D. J., Hayes, B., … Vinatzer, B. A. (2015). Genome-enabled phylogeographic investigation of the quarantine pathogen *Ralstonia solanacearum* race 3 biovar 2 and screening for sources of resistance against its core effectors. *Phytopathology*, *105*(5), 597–607. https://doi.org/10.1094/PHYTO-12-14-0373-R

Clough, S. E., Jousset, A., Elphinstone, J. G., & Friman, V. P. (2022). Combining in vitro and in vivo screening to identify efficient *Pseudomonas* biocontrol strains against the phytopathogenic bacterium *Ralstonia solanacearum*. *MicrobiologyOpen*, *11*(2), e1283. https://doi.org/10.1002/mbo3.1283

Compant, S., Duffy, B., Nowak, J., … Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: Principles, mechanisms of action, and future prospects. *Applied and Environmental Microbiology*, *71*(9), 4951–4959. https://doi.org/10.1128/AEM.71.9.4951-4959.2005

Crabbé, A., Jensen, P. O., Bjarnsholt, T., & Coenye, T. (2019). Antimicrobial tolerance and metabolic adaptations in microbial biofilms. *Trends in Microbiology*, *27*(10), 850–863. https://doi.org/10.1016/j.tim.2019.05.003

Cronin, D., Moenne-Loccoz, Y., Fenton, A., … O'gara, F. (1997). Role of 2,4-diacetylphloroglucinol in the interactions of the biocontrol pseudomonad strain F113 with the potato cyst nematode *Globodera rostochiensis*. *Applied and Environmental Microbiology*, *63*(4), 1357–1361. https://doi.org/10.1128/aem.63.4.1357-1361.1997

Elphinstone, J. G., Hennessy, J., Wilson, J. K., & Stead, D. E. (1996). Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. *Bulletin OEPP/EPPO Bulletin*, *26*(3–4), 663–678.

EPPO. (2021). *Ralstonia solanacearum species complex*. EPPO datasheets on pests recommended for regulation. https://gd.eppo.int

Farnham, E. M. (2022). *Exploring the phenotypic and genetic diversity of a bacterial plant pathogen, the Ralstonia solanacearum species complex (RSSC)* [Doctoral dissertation, University of York].

Fravel, D. R. (1988). Role of antibiosis in the biocontrol of plant diseases. *Annual Review of Phytopathology*, *26*(1), 75–91. https://doi.org/10.1146/annurev.py.26.090188.000451

Gabriel, D. W., Allen, C., Schell, M., … González, E. T. (2006). Identification of open reading frames unique to a select agent: *Ralstonia solanacearum* race 3 biovar 2. *Molecular Plant-Microbe Interactions*, *19*(1), 69–79.

Geller, A. M., & Levy, A. (2023). What I cannot create, I do not understand: Elucidating microbe–microbe interactions to facilitate plant microbiome engineering. *Current Opinion in Microbiology*, *72*, 102283. https://doi.org/10.1016/j.mib.2023.102283

Genin, S., & Denny, T. P. (2012). Pathogenomics of the *Ralstonia solanacearum* species complex. *Annual Reviews of Phytopatholgy*, *50*, 67–89. https://doi.org/10.1146/annurev-phyto-081211-173000

Geudens, N., & Martins, J. C. (2018). Cyclic lipodepsipeptides from Pseudomonas spp–biological Swiss-army knives. *Frontiers in Microbiology*, *9*, 1867. https://doi.org/10.3389/fmicb.2018.01867

Gillespie, S. H. (2002). Evolution of drug resistance in *Mycobacterium tuberculosis*: Clinical and molecular perspective. *Antimicrobial Agents and Chemotherapy*, *46*(2), 267–274. https://doi.org/10.1128/AAC.46.2.267-274.2002

Granato, E. T., Meiller-Legrand, T. A., & Foster, K. R. (2019). The evolution and ecology of bacterial warfare. *Current Biology*, *29*(11), R521–R537. https://doi.org/10.1016/j.cub.2019.04.024

Haas, D., & Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Reviews Microbiology*, *3*(4), 307–319. https://doi.org/10.1038/nrmicro1129

Haas, D., & Keel, C. (2003). Regulation of antibiotic production in root-colonizing Pseudomonas spp and relevance for biological control of plant disease. *Annual Reviews of Phytopathology*, *41*, 117–153. https://doi.org/10.1146/annurev.phyto.41.052002.095656

Hansen, M. L., Wibowo, M., Jarmusch, S. A., … Jelsbak, L. (2022). Sequential interspecies interactions affect production of antimicrobial secondary metabolites in *Pseudomonas protegens* DTU91. *The ISME Journal*, *16*(12), 2680–2690. https://doi.org/10.1038/s41396-022-01322-8

Hayes, M. M., Dewberry, R. J., Babujee, L., … Allen, C. (2022). Validating methods to eradicate plant-pathogenic *Ralstonia* strains reveals that growth in planta increases bacterial stress tolerance. *Microbiology Spectrum*, *10*(6), e0227022. https://doi.org/10.1128/spectrum.02270-22

Hayward, A. C. (1991). Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. *Annual Review of Phytopathology*, *29*, 65–87. https://doi.org/10.1146/annurev.py.29.090191.000433

Hu, J., Wei, Z., Friman, V. P., … Jousset, A. (2016). Probiotic diversity enhances rhizosphere microbiome function and plant disease suppression. *MBio*, *7*(6), 10–1128.

Humair, B., González, N., Mossialos, D., … Haas, D. (2009). Temperature-responsive sensing regulates biocontrol factor expression in *Pseudomonas fluorescens* CHA0. *The ISME Journal*, *3*(8), 955–965. https://doi.org/10.1038/ismej.2009.42

Jousset, A., Lara, E., Wall, L. G., & Valverde, C. (2006). Secondary metabolites help biocontrol strain *Pseudomonas fluorescens* CHA0 to escape protozoan grazing. *Applied and Environmental Microbiology*, *72*(11), 7083–7090. https://doi.org/10.1128/AEM.00557-06

Kaczmarek, A., Mackenzie, K., Kettle, H., & Blok, V. C. (2014). Influence of soil temperature on *Globodera rostochiensis* and *Globodera pallida*. *Phytopathologia Mediterranea*, *53*(3), 396–405.

Khokhani, D., Lowe-Power, T. M., Tran, T. M., & Allen, C. (2017). A single regulator mediates strategic switching between attachment/spread and growth/virulence in the plant pathogen *Ralstonia solanacearum*. *MBio*, *8*(5), 10–1128.

Kidarsa, T. A., Goebel, N. C., Zabriskie, T. M., & Loper, J. E. (2011). Phloroglucinol mediates cross-talk between the pyoluteorin and 2, 4-diacetylphloroglucinol biosynthetic pathways in *Pseudomonas fluorescens* Pf-5. *Molecular Microbiology*, *81*(2), 395–414. https://doi.org/10.1111/j.1365-2958.2011.07697.x

Kieser, T., Bibb, M. J., Buttner, M. J., … Hopwood, D. A. (2000) *Practical streptomyces genetics*. (*Vol. 291*, p. 397). John Innes Foundation.

Knöppel, A., Näsvall, J., & Andersson, D. I. (2017). Evolution of antibiotic resistance without antibiotic exposure. *Antimicrobial Agents and Chemotherapy*, *61*(11), e01495–e01417. https://doi.org/10.1128/AAC.01495-17

Landa, B. B., Mavrodi, O. V., Raaijmakers, J. M., … Weller, D. M. (2002). Differential ability of genotypes of 2, 4-diacetylphloroglucinol-producing *Pseudomonas fluorescens* strains to colonize the roots

of pea plants. *Applied and Environmental Microbiology*, *68*(7), 3226–3237.

Li, E., de Jonge, R., Liu, C., … Jousset, A. (2021). Rapid evolution of bacterial mutualism in the plant rhizosphere. *Nature Communications*, *12*(1), 3829.

Lopatkin, A. J., Bening, S. C., Manson, A. L., … Collins, J. J. (2021). Clinically relevant mutations in core metabolic genes confer antibiotic resistance. *Science*, *371*(6531), eaba0862. https://doi.org/10.1126/science.aba0862

Ma, Z., Geudens, N., Kieu, N. P., … Höfte, M. (2016). Biosynthesis, chemical structure, and structure-activity relationship of orfamide lipopeptides produced by *Pseudomonas protegens* and related species. *Frontiers in Microbiology*, *7*, 382. https://doi.org/10.3389/fmicb.2016.00382

Mansfield, J., Genin, S., Magori, S., … Foster, G. D. (2012). Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular Plant Pathology*, *13*(6), 614–629. https://doi.org/10.1111/j.1364-3703.2012.00804.x

Nielsen, T. H., Sørensen, D., Tobiasen, C., … Sørensen, J. (2002). Antibiotic and biosurfactant properties of cyclic lipopeptides produced by fluorescent *Pseudomonas* spp from the sugar beet rhizosphere. *Applied and Environmental Microbiology*, *68*(7), 3416–3423. https://doi.org/10.1128/AEM.68.7.3416-3423.2002

Nion, Y. A., & Toyota, K. (2015). Recent trends in control methods for bacterial wilt diseases caused by *Ralstonia solanacearum*, *Microbes and environments*. *Japanese Society of Microbial Ecology*, *30*(1), 1–11.

Peyraud, R., Cottret, L., Marmiesse, L., … Genin, S. (2016). A resource allocation trade-off between virulence and proliferation drives metabolic versatility in the plant pathogen *Ralstonia solanacearum*. *PLoS Pathogens*, *12*(10), e1005939. https://doi.org/10.1371/journal.ppat.1005939

Pierson, III, L. S., Wood, D. W., Pierson, E. A., & Chancey, S. T. (1998). N-acyl-homoserine lactone-mediated gene regulation in biological control by fluorescent pseudomonads: Current knowledge and future work. *European Journal of Plant Pathology*, *104*(1), 1–9.

R Core Team. (2023). *R: A language and environment for statistical computing*. R foundation for Statistical Computing. https://www.R-project.org/

Raza, W., Ling, N., Yang, L., … Shen, Q. (2016). Response of tomato wilt pathogen *Ralstonia solanacearum* to the volatile organic compounds produced by a biocontrol strain *Bacillus amyloliquefaciens* SQR-9. *Scientific Reports*, *6*(1), 1–13.

Riley, M. S., Cooper, V. S., Lenski, R. E., … Marsh, T. L. (2001). Rapid phenotypic change and diversification of a soil bacterium during 1000 generations of experimental evolution. *Microbiology*, *147*(4), 995–1006. https://doi.org/10.1099/00221287-147-4-995

Wang, X., Wei, Z., Li, M., … Friman, V. P. (2017). Parasites and competitors suppress bacterial pathogen synergistically due to evolutionary trade-offs. *Evolution*, *71*(3), 733–746. https://doi.org/10.1111/evo.13143

Wang, X., Wei, Z., Yang, K., … Friman, V. P. (2019). Phage combination therapies for bacterial wilt disease in tomato. *Nature Biotechnology*, *37*(12), 1513–1520. https://doi.org/10.1038/s41587-019-0328-3

Weller, D. M. (2007). *Pseudomonas* biocontrol agents of soilborne pathogens: Looking back over 30 years. *Phytopathology*, *97*(2), 250–256. https://doi.org/10.1094/PHYTO-97-2-0250

Xue, Q. Y., Ding, G. C., Li, S. M., … Smalla, K. (2013). Rhizocompetence and antagonistic activity towards genetically diverse *Ralstonia solanacearum* strains—An improved strategy for selecting biocontrol agents. *Applied Microbiology and Biotechnology*, *97*(3), 1361–1371. https://doi.org/10.1007/s00253-012-4021-4

Yasmin, S., Hafeez, F. Y., Mirza, M. S., … Iqbal, M. (2017). Biocontrol of bacterial leaf blight of rice and profiling of secondary metabolites produced by rhizospheric *Pseudomonas aeruginosa* BRp3. *Frontiers in Microbiology*, *8*, 1895.