The plant host environment influences competitive interactions between bacterial pathogens

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Abstract
Bacteria compete for resources in diverse environments using an array of antagonistic strategies, including the production of narrow-spectrum protein antibacterials termed bacteriocins. Although significant research has focused on bacteriocin-mediated dynamics in culture environments, little research has explored bacteriocin-mediated dynamics within a host context, particularly in plant environments. Here, we show that a bacterial plant pathogen, 

Pseudomonas syringae pv. syringae (Psy),

expresses a bacteriocin both in culture and in leaf apoplast when co-inoculated with a bacteriocin-sensitive competitor,

P. syringae pv. phaseolicola (Pph).

Although there is an observable negative effect of the bacteriocin on the Pph population at most time points both in culture and in the leaf apoplast, a bacteriocin-mediated benefit to Psy was only observed when the producing strain was co-infiltrated at a low population frequency (1:9) into the leaf apoplast. At 6 days post-infiltration, Psy achieved an eightfold population increase compared to a bacteriocin-deficient mutant in the apoplast. No bacteriocin-mediated benefit for Psy was observed under the culture conditions tested. Additionally, we found that the bacteriocin-mediated benefit for Psy was dependent on the Type III Secretion System. Taken together, our results demonstrate that the fitness benefit of bacteriocin-mediated antagonism is influenced by interactions within the host plant.

INTRODUCTION

Microbial competition is pervasive throughout the microbiological world. Populations with overlapping niche requirements often engage in both direct (interference) and indirect (exploitation) competition (Ghoul & Mitri, 2016; Granato et al., 2019). Outcomes of microbial competition have numerous consequences at the population and community levels and can also be harmful for plant and animal hosts by causing disease. For bacteria, one of the most intensively studied forms of competition is interference competition mediated by the production of antimicrobials, including protein toxins called bacteriocins (Ghequire & De Mot, 2014; Kommineni et al., 2015; Majeed et al., 2011; Riley & Wertz, 2002). Most bacteriocins are narrow spectrum within an individual species, where they target strains closely related to the producer (Mills et al., 2017; Riley & Chavan, 2007). A group of bacteriocins known as tailocins are multi-protein complex bacteriocins that are morphologically and evolutionarily related to the tails of Caudovirales bacteriophages (Ghequire & De Mot, 2014; Hockett et al., 2015; Scholl, 2017). Due to their large size, tailocins particles must be released via cell lysis resulting in a cost to the individual producing bacterium (Scholl, 2017).

In silico and in vitro work has demonstrated the benefit of bacteriocin production is dependent on the environmental context. Initial studies examined competition with two populations of a producer and a sensitive
strain in a physically unstructured environment such as liquid broth (Chao & Levin, 1981). When both populations are at equal frequencies, they can reach equilibrium as the sensitive population is able to exploit the resources made available from the killing activity by the producer (Durante & Levin, 1997). In this scenario, there is no fitness benefit for the producer as its population remains the same. However, when the environment is spatially structured (e.g. agar plate) the two species form microcolonies, which results in local interactions where bacteriocins only affect cells that are physically close to the producing cells, as well as resources made available by killing (Chao & Levin, 1981; Kerr, 2007). This allows the producer to preferentially gain a fitness benefit, an increase in population size, from the available resources and space.

Competition within host environments can add an additional level of complexity compared to a static lab culture setting, given the host’s potential to sense and respond to microbial invaders. In mice models, bacteriocin production by the human pathogen Salmonella enterica in the gut provides a competitive advantage against Escherichia coli, but only if S. enterica is able to induce inflammation (Nedialkova et al., 2014). The change in the gut environment results in both increased bacteriocin production by S. enterica as well as expression of the bacteriocin surface receptor in E. coli. Other in vivo studies in animal systems have also shown that bacteriocin production can result in the reduction or elimination of targeted populations (Corr et al., 2007; Kommineni et al., 2015; Sassone-Corsi et al., 2016; Yu et al., 2020), though it is not clear to what extent interaction with the animal host was important for the competitive outcomes in these studies. Indeed, there have been very few studies that have explicitly assessed the role of the host in affecting bacteriocin-mediated interactions.

Bacteriocins have also been shown to promote invasion of the producer into another population. Invasion into a sensitive population by six E. coli colicin producers was positive-frequency dependent (Riley & Gordon, 1999). When the number of E. coli producing cells increased, resulting in a higher toxin particle number, the time needed for invasion decreased. Whereas the growth of another E. coli bacteriocin producing population was negative-frequency dependent, in that bacteriocin production was beneficial when the producer’s population was small relative to the competitor (Chao & Levin, 1981). Furthermore, higher bacteriocin production rates aided invasion into a bacteriocin-sensitive population, especially at low initial cell frequencies (Ghazaryan et al., 2019). Contrary to these findings, modelling predicts a different outcome where the benefits for the producer at low frequency are reduced as resources liberated by bacteriocin killing will be as likely to benefit the sensitive population as it will the bacteriocin producing population (Inglis et al., 2009; Weber et al., 2014).

Beyond basic ecological questions, understanding the fitness benefit of bacteriocins has implications for the creation of biological control agents. For plant health, we have relied on antagonist mechanisms such as toxins and antibiotics; however, it could be useful to also think about when and where it is beneficial for the agent to antagonize a target pathogen. Bacteriocins are of increasing interest as they could reduce non-target effects as observed with chemicals (McEvoy, 1996; Montesinos, 2007). On the surface of plant cells within the apoplast, bacteria can form microcolonies of single or multiple species where they can interact and compete (Bogino et al., 2013; Morris & Monier, 2003). In addition, bacteria have to evade host defences to successfully populate using virulence factors or by in trans effector-mediated plant defence suppression for example (Dodds & Rathjen, 2010; Rufian et al., 2018; Singh & Singh, 2018; Xie et al., 2018). To date, there are few studies that have investigated the role of bacteriocin-mediated antagonism in a plant context, let alone in the apoplast, thus it is not clear how much dynamic interaction there was with the host (Dorosky et al., 2018; Godino et al., 2016; Hert et al., 2005; Li et al., 2020).

To investigate plant-associated bacteriocin-mediated competition, we used Pseudomonas syringae as a model, as it is possible for multiple distantly related strains of this species to infect the same plant host and many can produce bacteriocins to antagonize competitors (Hirano & Uppper, 2000; Holtmark et al., 2008). Here, we performed a series of in vitro co-infections and in planta co-infections over an 8-day period with P. syringae pv. syringae (Psy) and P. syringae pv. phaseolicola (Pph), both virulent plant pathogens that cause bacterial brown spot and halo blight in Phaseolus vulgaris (Common bean), respectively (Burkholder, 1926; van Hall, 1902). Psy is a generalist pathogen that can infect multiple hosts, whereas Pph is a specialist with a narrow host range of legume species (Baltrus et al., 2011; Morris et al., 2019). Key to this interaction is that Psy encodes a bacteriocin that targets Pph (Hockett et al., 2015). In this study, we sought to answer two related questions. First, under what ecological conditions is bacteriocin production beneficial for the producer? Second, how do host interactions influence the fitness benefits of bacteriocin production (i.e. an increased population size when in competition)? This work highlights the importance of understanding how host structure and activity influence microbial competition and is a critical step to improve disease suppression in plant and animal hosts.
RESULTS

Bacteriocins are expressed in vitro but there is no detectable fitness benefit for Psy at 1:1 and different co-inoculation ratios

To investigate whether bacteriocin-mediated antagonism provides a fitness benefit within an agar environment, we spotted individual or mixed strains (1:1) of either Psy (bacteriocin-producer) or Psy ΔRrbp (bacteriocin-deficient mutant; Hockett et al., 2015), and Pph (bacteriocin-sensitive) on KB agar. At several time points post-inoculation, the growing culture was sampled, and strains were enumerated (Table S1). Pph populations were reduced in co-inoculation with either Psy or Psy ΔRrbp at all time points in comparison to Pph-only [Figure 1(A)]. The population reduction was greater for Pph co-inoculated with Psy compared to Psy ΔRrbp, suggesting the bacteriocin was expressed and active under these culture conditions. Bacteriocin production did not provide a detectable fitness benefit in 1:1 co-inoculation in an agar setting due to no significant fitness differences between Psy or Psy ΔRrbp populations in individual or co-inoculations [Figure 1(B)]. Individual inoculation of the bacteriocin-deficient complement strain Psy ΔRrbp:Rrbp at 4 dpi seemed to be greater by threefold compared to Psy ΔRrbp; however, the statistical difference is only true in one of three independent experiments \( p \leq 0.04; \text{Figure S2(B)} \).

As bacteriocin production in 1:1 co-inoculation did not result in a fitness benefit for Psy, we sought to assess whether the population frequency influenced competition, as has been previously shown in other systems (Chao & Levin, 1981; Gordon & Riley, 1999; Inglis et al., 2009; Kerr, 2007). We altered the inoculation frequency between Psy strains and Pph to either 1:9 or 9:1. For example, ‘Psy minority’ would represent 1:9 (Psy:Pph), and vice versa for ‘Psy majority’ at 9:1 (Psy:Pph). In vitro Pph minority populations were reduced in co-inoculation with Psy or Psy ΔRrbp compared to Pph-only across all time points \( p \leq 0.0001; \text{Figure 1(C)} \). In Pph majority competitions, Pph growth with Psy and Psy ΔRrbp was not different to Pph-only [Figure 1(C)]. Somewhat unexpectedly, Psy minority reached a population equivalent to the Psy majority treatments by 4 dpi \( p \leq 0.0001; \text{Figure 1(D)} \).

FIGURE 1 Bacteriocin-mediated competition in vitro detrimental for sensitive strain, yet no fitness benefit for producer. Bacterial populations of Pph in (A) 1:1 and (C) 1:9 initial starting frequencies (Psy:Pph), and Psy strains in (B) and (D), respectively, are shown across 8 days post-inoculation. Data points represent four replicates from three independent experiments and error bars indicate standard error of the mean. Average values with the same letter are not significantly different by Tukey’s HSD test \( p \leq 0.05 \).
trend was maintained at 6 and 8 dpi. As expected, the $Psy\Delta Rrbp:Rrbp$ population behaved similarly to $Psy$ and $Psy\Delta Rrbp$ in both individual and mixed inoculations [Figure S2(D)]. Taken together, in vitro, there was no detectable benefit to bacteriocin production for $Psy$, yet a negative effect of bacteriocins on $Pph$ is clearly observed. $Psy$ is competitively superior to $Pph$ in 1:1 plant co-infiltration regardless of bacteriocin production

To determine whether the leaf apoplast environment affects the competitive interactions between $Psy$ and $Pph$, we infiltrated common bean leaves with either individual or 1:1 mixed inocula of the same strains used in the in vitro assay. Similar to our in vitro results, we found that co-infiltration with $Psy$ resulted in a 10-fold greater reduction of the $Pph$ population than co-infiltration with $Psy\Delta Rrbp$ [$p \leq 0.0001$; Figure 2(A)]. This effect, however, was only observed at 4 dpi. There were no statistical differences after this timepoint, where the $Pph$ population was suppressed by a similar amount by both $Psy$ and $Psy\Delta Rrbp$. The complement strain $Psy\Delta Rrbp:Rrbp$ showed a similar reduction of $Pph$ at 4 dpi, suggesting the $Pph$ reduction in co-infiltrations is due to bacteriocin production [$p \leq 0.0001$: Figure S3(A)].

For $Psy$, there was no difference in population growth for $Psy$-only compared to $Psy\Delta Rrbp$-only at all dpi [Figure 2(B)]. There was, however, a sevenfold reduction in population size of $Psy$ and $Psy\Delta Rrbp$ in 1:1 co-infiltration at 4 dpi compared to $Psy$-only and $Psy\Delta Rrbp$-only, respectively ($p \leq 0.0001$). Therefore, 1:1 co-infiltration with a sensitive strain shows that bacteriocin production provided no fitness benefit for $Psy$.

**At low frequency, bacteriocin production provides $Psy$ a fitness benefit when competing with $Pph$ in the apoplast**

The negative effect of the bacteriocin production on $Pph$ minority was apparent at all time points, with no effects on $Pph$-majority populations [Figure 2(C)]. Notably, there was an 80-fold reduction for co-infiltrated $Pph$
minority with *Psy* at 4 dpi compared to *Pph* minority co-infiltration with *Psy ΔRrbp* \(p \leq 0.0001\). The differences in population to *Pph*-only were greater for *Pph* minority with *Psy* (6000-fold) in comparison to co-infiltration with *Psy ΔRrbp* (80-fold; \(p \leq 0.0001\)). The differences between co-infiltrated *Pph* minority populations decrease at 6 and 8 dpi, yet the population sizes of co-infiltrated compared to *Pph*-only remained fairly similar to levels at 4 dpi \(p \leq 0.0001\).

When *Psy* minority is co-infiltrated with *Pph* there is a statistical eightfold increase compared to co-infiltrated *Psy ΔRrbp* minority at 6 dpi [Figure 2(D); \(p \leq 0.0001\)]. Both *Psy* minority and *Psy ΔRrbp* minority performed equivalently at 4 or 8 dpi, indicating this was the first observation that bacteriocin production is beneficial when faced with a dominant sensitive population. The *Psy ΔRrbp* minority presented between a 5- to 10-fold reduction at all time points to *Psy ΔRrbp*-only \(p \leq 0.0001\) for all comparisons). The complement *Psy ΔRrbp::Rrbp* minority population level was similar to *Psy* minority indicating the population increase is due to bacteriocin production [Figure S3(B)]. There were no differences between *Psy* majority and *Psy ΔRrbp* majority when co-inoculated with *Pph* [Figure 2(D)]. Additionally, *Psy* majority strains exhibited a decrease in population relative to the *Psy*-only infiltration at 4 dpi but maintained roughly equivalent populations at 6 and 8 dpi. Overall, *Psy* starting at a low cell frequency (in the minority) provided a bacteriocin-mediated fitness benefit.

**Psy virulence is required for in planta bacteriocin-mediated effects**

To investigate the role of virulence in pathogen–host interactions, a mutation of the *hrcC* gene, a structural component of the Type III Secretion System (T3SS), was introduced into *Psy*. *hrcC* mutants are impaired in their ability to suppress plant defences, and thus incapable of causing disease (Deng et al., 1998; Hirano et al., 1999). The *Pph* population in planta was not affected during 1:1 co-infiltration with either *Psy ΔhrcC* or *Psy ΔRrbp::ΔhrcC* and was able to maintain populations comparable to *Pph*-only infiltration for all dpi [Figure 3(A)]. This was opposite to in vitro
competition, where Pph co-inoculated with Psy ΔhrC at 4 and 6 dpi was reduced by 10-fold compared to the 100-fold reduction at 8 dpi with Psy ΔRrgb/ΔhrC [Figure 3(C)]. In comparison to Pph-only, a 100-fold and 10-fold detriment occurred for Pph in co-inoculation with Psy ΔhrC and Psy ΔRrgb/ΔhrC, respectively.

No differences were observed between Psy ΔhrC and Psy ΔRrgb/ΔhrC populations in planta during 1:1 co-infiltration with Pph [Figure 3(B)]. Yet, these populations were partially rescued when co-infiltrated with Pph by an average increase of 8- to 20-fold at 6 and 8 dpi compared to Psy ΔhrC-only and Psy ΔRrgb/ΔhrC-only (p ≤ 0.0001). Both individual and co-infiltrations of Psy ΔhrC and Psy ΔRrgb/ΔhrC were reduced by 50- to 100-fold from 4 to 8 dpi compared to Pph-only infiltration (p ≤ 0.0001). Whereas in vitro competition showed no differences between any individual and mixed Psy strains [Figure 3(D)]. These results show that virulence is required for Psy to dominate the co-infection environment, as well as to gain a fitness benefit from bacteriocin production.

**No bacteriocin-mediated fitness benefit for Psy in co-infiltration with avirulent sensitive strain**

To identify if Psy could gain a fitness benefit in co-infections with an avirulent sensitive strain, the hrpL gene, required for the activation of the hrp/hr locus responsible for T3SS expression, was knocked-out in Pph (Hockett et al., 2015; Ortiz-Martín et al., 2010). The Pph ΔhrpL populations in planta were reduced in both individual (1000-fold) and co-infections (between 10- to 100-fold) compared to Pph-only from 4 dpi [p ≤ 0.0001; Figure 4(A)]. For all timepoints the co-infiltration of Pph ΔhrpL with Psy resulted in a 9- to 30-fold increase to Pph ΔhrpL-only (p ≤ 0.0001). However, the population of Pph ΔhrpL co-infiltration with Psy was lower than co-infiltration with Psy ΔRrgb at 4 and 8 dpi (p ≤ 0.0302). In vitro competition resulted in a similar trend with co-inoculated Pph ΔhrpL presenting a 20-fold reduction with Psy compared to Psy ΔRrgb at 4 dpi and reduced by 70- to 80-fold at 6 and 8 dpi [p ≤ 0.0001; Figure 4(C)]. By 8 dpi, the population...
of \textit{Pph} \(\Delta hrpL\) co-inoculated with \textit{Psy} \(\Delta Rrbp\) is equal to \textit{Pph} \(\Delta hrpL\)-only.

The co-infiltration of \textit{Psy} with \textit{Pph} \(\Delta hrpL\) was not statistically different in \textit{ planta} to co-infiltrated population of \textit{Psy} \(\Delta Rrbp\), and both \textit{Psy}-only and \textit{Psy} \(\Delta Rrbp\)-only [Figure 4(B)]. However, in vitro co-inoculated \textit{Psy} was statistically reduced by threefold compared to \textit{Psy}-only \([p \leq 0.033; \text{ Figure 4(D)}]\). Together these results suggest a virulent producer does not benefit from bacteriocin-mediated competition with an avirulence sensitive population.

**DISCUSSION**

In this study, we sought to understand how interactions with a host plant affect bacteriocin-mediated competition between two bacterial plant pathogens. Overall, our in vitro results did not show a bacteriocin-mediated fitness benefit (i.e. an increased population size) for the bacteriocin producer, \textit{Psy}, at any starting frequency when competing with the sensitive strain, \textit{Pph}. Conversely, \textit{ in planta} co-infiltrations did show a bacteriocin-mediated fitness benefit for \textit{Psy} when at an initially low frequency. \textit{Pph}, however, suffered from bacteriocin-mediated inhibition during both in vitro and \textit{ in planta} co-inoculation. Intriguingly, the \textit{ in planta} benefit to \textit{Psy} and detriment to \textit{Pph} occurred at specific time points and were not maintained consistently across all time points. Additionally, virulence aided \textit{Psy} bacteriocin-mediated suppression of \textit{Pph} in the plant environment. These results indicate that bacteriocin-mediated interactions within a host plant are influenced by host physiology and pathogen virulence over the course of an infection.

Previous bacteriocin antagonism studies have been performed using computer models or laboratory systems, showing that a fitness benefit for the toxin-producing population is dependent on the environment (Chao & Levin, 1981; Kerr, 2007; Majeed et al., 2011). Our results suggest that when at parity or in the majority there was no bacteriocin-mediated fitness benefit in vitro for \textit{Psy} compared to \textit{Psy} \(\Delta Rrbp\) across 8 dpi. It is likely that the sampling of the entire colony is a global measurement of the cumulative effects of local interactions between cells that might mask a fitness benefit that is localized to the colony periphery. The detriment was not a complete elimination of \textit{Pph} as it is hypothesized that primarily sensitive cells at the edges are affected, with the cells near the centre of the micro-colony being able to persist (Kerr, 2007). Both \textit{Psy} minority strains are also able to overcome the initial low frequency (regardless of bacteriocin killing) compared to the \textit{Pph} minority indicating \textit{Psy} possesses some additional method of competitive advantage over \textit{Pph}.

Differences in apoplast spatial structure, available resources and host compatibility create a dynamic host environment for two pathogens to compete that is more complex than an agar plate (Dangl & Jones, 2001; Farvardin et al., 2020; O’Leary et al., 2016; Rico & Preston, 2008). In 1:1 co-infiltration, the outcome of \textit{ in planta} competition was similar to the in vitro competition where there was no benefit for the bacteriocin producer but there was a bacteriocin mediated detriment to \textit{Pph}, similar to the effects Li et al. (2020) showed for competition between \textit{P. syringae} pv. \textit{tomato} and \textit{P. syringae} pv. \textit{lachrymans} (Li et al., 2020). However, the detriment was not maintained from 6 dpi onwards, potentially indicating the effect of bacteriocin production in the apoplast is limited either by changes in behaviour of \textit{Pph} or changes in the apoplast environment, or both. Previous work has suggested that both sensitive and producing populations are able to coexist through spatial partitioning (Czárán & Hoekstra, 2003; Kerr, 2007), which may occur in the leaf apoplast.

Our results indicate that bacteriocin production is beneficial in a negative-frequency dependent manner, where production is favoured when the population is low (Kerr, 2007; Müller et al., 2019). In our case, this fitness benefit was not observed immediately post-inoculation and occurred once the initial \textit{Psy} minority had a high population level. Importantly, our results showed that the \textit{Pph} population was significantly reduced by bacteriocin-mediated killing at 4 dpi and that the \textit{Psy} population is suppressed when co-inoculated with \textit{Pph} compared to \textit{Psy}-only at the same time point. Taken together, these results indicate that it should have been possible to observe a bacteriocin-mediated benefit for \textit{Psy} at 4 dpi. We also considered the use of the competitive index (CI) to present the fitness benefit, since we have paired populations of \textit{Pph} and \textit{Psy} strains for each treatment. We believe, however, that such calculations would be misleading as the CI will certainly show \textit{Psy} performing better compared to \textit{Psy} \(\Delta Rrbp\), but this difference would, in nearly all cases, result from less killing of \textit{Pph} by \textit{Psy} \(\Delta Rrbp\) rather than any increase in the \textit{Psy} population compared to \textit{Psy} \(\Delta Rrbp\). This observation also occurs about the same time that disease symptoms for \textit{Pph} were distinctly identifiable in individual infiltration (e.g. water-soaking and yellowing). Therefore, we hypothesize that during co-infiltration \textit{Pph} can gain greater access to host nutrients at the height of disease progression increasing its population, and the bacteriocin production of \textit{Psy} is able to overcome this growth whereas \textit{Psy} \(\Delta Rrbp\) cannot resulting in the reduction of \textit{Psy} \(\Delta Rrbp\) population at 6 dpi.

Bacterial pathogens must contend with the host plant defences to enable establishment and proliferation of their populations. Pathogenic bacteria can use the T3SS encoded by the \textit{hrp} and \textit{hrc} genes to suppress the plants response in nearby plant cells (Alfano et al., 2000; Arnold et al., 2003). During \textit{ in planta} infiltrations both \textit{Psy} \(\Delta hrcC\) and \textit{Psy} \(\Delta Rrbp/\Delta hrcC\) populations were greatly reduced compared to \textit{Psy}.
When co-infiltrated, however, T3SS mutant strains received an *in trans* benefit from the virulent *Pph* strain. This has been observed in other work with virulent strains of *P. syringae* which relied on the proximity of virulent strains to reduce the plant cells effector-triggered immunity (Macho et al., 2007; Omer & Wood, 1969; Ruffan et al., 2018). Similarly, *Pph ΔhrpL* benefitted from co-infiltration with virulent *Psy*. There was also no detriment for *Pph* in the presence of *Psy ΔhrcC*, indicating that *Psy* virulence is required for a bacteriocin-mediated effect on *Pph*.

While previous research has focused mainly on the outcomes of bacteriocin-mediated antagonism in vitro, we show that such outcomes may not be directly translated into a host plant environment. Our findings show that under certain frequency and temporal conditions bacteriocin production can promote *Psy* fitness while targeting the sensitive strain population. Further research is needed to elucidate the exact spatial distribution of the infiltrated bacteria, such as the use of fluorescent microscopy, alongside measuring the rates of bacteriocin production in the apoplast. Bacteriocin-mediated killing does not necessarily equate to a bacteriocin-mediated fitness benefit. We suggest that this dichotomy applies to past and current biological control research, where the objective typically is to limit the effects of the pathogen but there was no examination of the benefit of the agent to proliferate and maintain present in the field (Fravel, 1988).

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**CONFLICT OF INTEREST**
All authors declare no conflict of interest.

**DATA AVAILABILITY STATEMENT**
Data available on request from the authors.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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