Antagonistic interactions of soil pseudomonads are structured in time

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Abstract

Social interactions have been invoked as potential major selective forces structuring natural microbial communities and thus may help explain the astonishing bacterial diversity of natural ecosystems. Here, we investigate the prevalence and structure of exotoxin-mediated antagonistic interactions among free-living soil *Pseudomonas* strains collected over the course of two years at distances of up to one kilometer. Unlike some previous studies on antagonistic interactions among natural isolates, we found the prevalence of exotoxin-mediated inhibitions to be relatively low. When present, antagonistic interactions show a weakly negative relationship with genetic relatedness and metabolic similarity. Intriguingly, isolates sampled from the same growing season were significantly more likely to inhibit each other than they were to inhibit isolates from different growing seasons. Exotoxin-mediated antagonistic interactions between soil pseudomonads thus seem to be structured in time but do not appear to be a major selective force structuring free-living soil bacterial communities of soil pseudomonads.

Introduction

The immense diversity of natural bacterial communities (Fierer & Jackson, 2006, Roesch *et al.*, 2007, Youssef & Elshahed, 2009, Kirchman *et al.*, 2010, Locey & Lennon, 2016) is at odds with the expectation that strong natural selection should eliminate all but the fittest type from any given environment, a phenomenon that has come to be known as the paradox of diversity (Kassen & Rainey, 2004, Lerat *et al.*, 2005, Li & Stevens, 2010). While strong divergent selection imposed by abiotic variation in conditions of growth across sites plays a role in supporting this diversity (Belotte *et al.*, 2003, Kraemer & Kassen, 2015), it has been suggested that social interactions, both cooperative and antagonistic, are also important (Czaran *et al.*, 2002, Riley & Wertz, 2002, Abrudan *et al.*, 2012, Mitri & Foster, 2013, Kelsic *et al.*, 2015,). This interpretation gains support from laboratory studies documenting the maintenance of diversity associated with antagonistic (Kerr *et al.*, 2002, Becker *et al.*, 2012, Rendueles *et al.*, 2015) and cooperative (Rainey & Rainey, 2003, Griffin *et al.*, 2004, Diggle *et al.*, 2007, Kohler *et al.*, 2010, Jousset *et al.*, 2013, Inglis *et al.*, 2016) interactions in bacteria, and the observation that a range of social interactions can evolve rapidly in the laboratory (Fiegna *et al.*, 2006, Hansen *et al.*, 2007, Zhang *et al.*, 2009).

Evaluating the hypothesis that social interactions underpin microbial diversity in natural environments has proven challenging, however, because we know relatively little about the prevalence and nature of social interactions outside the lab. The study of cooperative interactions in natural systems has largely focused on different strains of the same species (e.g. Stefanic & Mandic-Mulec, 2009, Kraemer and Velicer, 2011, Stefanic *et al.*, 2015; but see: Vetsigian *et al.*, 2011 and Foster and Bell, 2012), likely because of the presumed importance of kin selection in the evolution of cooperation. Antagonistic interactions, on the other hand, have been examined across a wider range of taxonomic levels in bacteria and appear to be highly variable, with a prevalence of 2-90% depending on the study (Vetsigian *et al.*, 2011, Cordero *et al.*, 2012, Foster and Bell, 2012). Any general statement about the impact of social interactions on microbial diversity is therefore difficult to make.

Here, we provide insight into the prevalence of exotoxin-mediated antagonistic interactions between free-living *Pseudomonas* isolates from forest soil samples. Like other bacteria, pseudomonads produce a wide variety of exotoxic agents, including broad-spectrum antibiotics, bacteriocins, and other anti-competitor molecules (Michel-Briand & Baysse,

2002, Riley & Wertz, 2002, Allen *et al.*, 2010). Exotoxins are often costly to produce and release, and so are not expected to be employed as all-purpose weapons. Rather, exotoxin production should be targeted towards genetically distinct lineages that compete most strongly with a given producing strain. The logic here is that antagonistic effects against isogenic clones produce no fitness benefit to the producer (because they either self-inhibit or share resistance mechanisms) while those against genetically very divergent strains are wasted because the producer and victim strain do not actually compete for the same resources. Consistent with this idea, several studies have reported that antagonistic interactions are preferentially directed at strains that have high niche-overlap with the producer strain (Cordero *et al*., 2012, Schoustra *et al*., 2012, Kinkel *et al.*, 2014, Bruce *et al*., 2017) and are most effective on genotypes of intermediate phylogenetic distance from the producer (Cordero *et al.*, 2012, Schoustra *et al.*, 2012). A number of studies have emphasized, in addition, that the complex dynamics among producer, sensitive, and resistant strains may be linked to the maintenance of biodiversity (Czaran *et al*., 2002, Kerr *et al*., 2002, Abrudan *et al.*, 2012, Inglis *et al*., 2016).

Our study pays particular attention to the spatial and temporal structure of the community. Because competition is expected to be the strongest between co-occurring strains (Long & Azam, 2001, Freilich *et al.*, 2011, Kinkel *et al*., 2014), we expect that antagonistic interactions would be strongest among genetically distinct isolates in close spatial and temporal proximity to each other. There is little data available to evaluate this prediction. While it is widely recognized that the spatial structure of antagonistic interactions can play an important role in structuring the diversity of microbial communities (e.g. Czaran *et al*., 2002, Kerr *et al*., 2002, Hawlena *et al*., 2012, Perez-Gutierrez *et al*., 2013, Kelsic *et al*., 2015, Bruce *et al*., 2017), the spatial scale of these interactions in some studies can be surprisingly large, on the order of meters, and even hundreds of meters, apart (Hawlena *et al*., 2010*a*, Perez-Gutierrez *et al.*, 2013, Bruce *et al*., 2017). This result suggests that either there is strong selection to evolve resistance between co-occurring strains (Bruce *et al*., 2017) or such extensive dispersal that antagonism shows no spatial structure (Bell, 2010). Studies on how antagonism is structured in time remain rare (but see: Wilson *et al*., 2009, Ghoul *et al*., 2015).

Here, we explore the genetic, spatial, and temporal structure of exotoxin-mediated antagonistic interactions among a subset of isolates from a collection of forest soil-derived bacteria from Western Québec, Canada. These isolates have been previously shown to be locally adapted to their environment in space and time (Kraemer & Kassen, 2015 & 2016),

although the selective pressures causing local adaptation were not investigated. In the previous experiment (Kraemer & Kassen, 2015), fitness was measured in soil infusion media, growth media derived from the same soil sample that the isolates originated from (Belotte *et al.*, 2003). Soil infusion media consists of a filter-sterilized soil-water infusion. Thus, among numerous abiotic components present in soil infusion media such as nutrient ion composition or pH, bacteriocins and other exotoxins, which are small enough to pass through the filter, could constitute a biotic component of selection responsible for the patterns observed previously. In the present work, we directly test this hypothesis by conducting inhibition assays between 18 *Pseudomonas* soil isolates sampled at different times and locations within the same environment. We set out to answer the following questions: 1) How frequent is exotoxin-mediated inhibition among isolates and how likely is inhibition to cause the patterns of adaptation observed previously? 2) Are patterns of inhibition structured in time or space? 3) How does the inhibition probability correlate with genetic relatedness and metabolic similarity?

Materials and Methods

Bacterial isolates

We studied a subset of the 243 isolates from the Gatineau Park Reserve (Gatineau, Québec, Canada) that were obtained from soil samples collected along transects according to the protocol outlined previously (Kraemer & Kassen, 2015 & 2016) during two eight month periods (growth seasons) in 2012 and 2013. Briefly, at each sampling site we carefully brushed away leaf litter and then scooped approximately 40 g of topsoil into a sterile falcon tube. Tubes were transferred into the lab and processed within 24 hours. All soil samples were passed through a sterile 3 x 3 mm metal sieve to remove stones and leaf litter. Approximately 0.5 g of sieved soil was added to 15 mL of sterile ddH_2O, vortexed and then incubated at room temperature for 15 minutes. The resulting soil suspension was subsequently diluted onto Pseudomonas isolation agar (Thermo Fisher Scientific, Lenexa, KS, USA) and incubated at 28 ˚C for 48 hours. After this incubation period, plates were very briefly exposed to UV light and the most common fluorescent phenotype picked, yielding one isolate per soil sample. Isolates were streaked three times onto King's Broth (KB) agar to ensure monoclonality before mixing them with glycerol to a final concentration of 20% and frozen storage at -80 ˚C.

Isolate names consist of their respective km-scale sampling sites (C, N or R, where C an N are approximately 600 meters apart from each other and 1000 meters apart from R, Fig. 1) combined with the meter marker they were sampled at (1, 6, or 12). The spatial information is followed by the year and the abbreviated month of sampling. For example, isolate C6-2012-Sep was sampled at km-scale site C, meter marker 6 in September 2012. Out of the 243 isolates sampled, 65 were identified as *Pseudomonas* via 16S rRNA sequencing. Among these strains, eighteen isolates were randomly selected for this study. The closest taxonomic affiliation of each strain can be found in Supplemental Table 1.

Inhibition assays

We tested for exotoxin-mediated inhibition between all pairwise combinations of isolates, giving a total of 342 comparisons (including self-comparisons). Due to the large total number of tests, we randomly allocated the 18 chosen isolates to two test groups of nine. We performed inhibition assays in four blocks of 81 pair-wise comparisons: group 1 against group 1, group 1 against group 2, group 2 against group 1 and group 2 against group 2. Each inhibition assay was replicated three times.

For each block of the inhibition assay, we inoculated each isolate into five mL of lysogeny broth (LB) media and incubated the cultures under aerobic shaken conditions (150 rpm) at 28 °C for 48 h to yield dense, stationary phase cultures. To isolate exotoxins, 1.2 mL of each potential producer culture was centrifuged at 10,000 rpm for ten minutes. Subsequently, one mL of the resulting supernatant was filtered through a sterile 0.22μm filter to remove suspended cells.

Inhibition assays were performed on LB hard agar plates (LB with 15 g/L of agar), which were divided into nine equal-sized wedges. For each plate, we inoculated three mL of molten LB soft agar (LB with 7.5 g/L of agar) with 100 μL of exponentially growing culture of the focal test isolate and poured the agar evenly over the plate. After the agar solidified, 15 μL of supernatant from each of the nine potential inhibitor isolates were spotted onto the overlay in individual wedges. Plates were allowed to dry before incubation for 48 h at 28°C.

After the incubation period, we inspected plates for evidence of growth inhibition in each wedge. When observed, inhibition ranged from faint circles of sparser growth to complete lack of growth in the spotted area. Where inhibition was inconsistent between

replicate blocks, we report the result observed in the majority of cases (Supplemental Table 2).

Estimation of genetic distance and metabolic dissimilarity

Pair-wise estimates of genetic distance between isolates were based on PCR and Sanger-sequencing of a fragment of the 16S rRNA gene ranging from position 142 to 701 (Kraemer & Kassen, 2015). All sequence data is available under Genbank accession numbers KM819176-KM819461 and KX500157-KX500188. Genetic distances were estimated utilizing the distance matrix option of the RaXML v8.2 algorithm, assuming a GTR model with GAMMA distributed rate heterogeneity (Stamatakis, 2014). The protocol for the estimation of metabolic dissimilarity was adapted from Schoustra *et al*. (2012). We measured metabolic dissimilarity based on the ability of each isolate to use 95 different carbon sources using GN2 MicroPlates (Biolog, Hayward, CA, USA). We grew the isolates in liquid LB media for 48 h and starved them for two hours by adding 20 µL of exponential phase culture to 20 mL of minimal salts medium (6.78 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl) before inoculating the GN2 MicroPlates. Optical density readings of each plate were taken at 590 nm immediately after inoculation and after 48 h of incubation at 28 °C. The difference in OD after incubation is a measure of the ability of an isolate to use a particular resource. We determined metabolic similarity between isolates as the pair-wise Pearson's correlation coefficient of the OD change. Metabolic dissimilarity is calculated as one minus the correlation coefficient.

Causative agent of exotoxicity

To confirm inhibition by proteinaceous agents, we repeated the inhibition assay as previously described, but heat-treated half of the supernatant solution at 100 °C for 20 seconds. Proteinaceous agents of inhibition should be inactivated by heat treatment, while untreated supernatant is expected to cause a zone of inhibition. Heat-treated supernatants did not produce inhibition in any case. To exclude the possibility of inhibition by bacteriophages, we transferred zones of inhibition on overlays from the previous assay into exponential liquid culture of the recipient isolate and grew them for 24 h in liquid LB. The cell-free supernatant was extracted as before and spotted on the recipient isolate in an inhibition assay. Observing

inhibition in this assay would implicate bacteriophages, as they would have multiplied in the host population. No inhibition was observed in these assays. Lastly, we observed no evidence of self-inhibition, suggesting that non-specific toxins like waste products are not responsible for our results.

Statistical analysis

We conducted all statistical analyses using R version 3.3.1 (R Development Core Team, 2009). We used the glmer function implemented in the lme4 package (Lamprianou, 2013) to calculate logistic regression models with the presence or absence of inhibition for each test pair as the dependent variable and genetic distance, temporal distance, metabolic dissimilarity, and whether or not isolates from the pair were from the same or different sampling seasons and km-scale sites as explanatory variables. Isolate identities of both the producer and the recipient strain were fitted as random effects to control for non-independence of observations. We performed Mantel tests to detect covariance between the distance measures using the ade4 package (Popescu *et al.*, 2012).

Results

How frequent is inhibition between isolates?

We tested for inhibition by exotoxins in a total of 324 (306 excluding self-inhibition tests) pairwise comparisons between 18 soil isolates. Our results, shown in figure 2, reveal that just 17 of the 306 non-self inhibition assays (~6%) resulted in growth inhibition, and no instances of self-inhibition. The majority of interactions resulting in inhibition (15 out of 17 non-self interactions) involved isolates that were either a producer or a victim, but not both. We detected a total of seven toxin producers within our dataset of 18 strains, resulting in a frequency of 39% active exotoxin-producers. On average, a single sensitive isolate was inhibited by two producer isolates. Two isolates (C6-2012-Sep and R6-2012-Aug) were notable exceptions, being proficient toxin producers targeting three to four isolates, while only being inhibited by each other.

Genetic and ecological divergence among isolates in space and time

We investigated the genetic and ecological divergence among isolates across space and time in our isolate collection. Specifically, we measured genetic divergence as genetic distance, based on a fragment of the 16S rRNA sequences, and ecological divergence as metabolic dissimilarity calculated from carbon resource use overlap between pairs of strains. We regressed each of these measures against the spatial and temporal distances between the sites from which the isolates were obtained. Metabolic dissimilarity was weakly but significantly positively correlated with temporal sampling distance (Mantel test between the distance between sampling time points of isolates and metabolic dissimilarity $r = 0.03$, $p < 0.05$). This result implies that isolates sampled closer together in time are ecologically more similar than those sampled from disparate time points. In contrast, we did not detect any indication of a temporal structure to genetic distance, nor any correlations between spatial distance and either genetic distance or metabolic dissimilarity in this collection (Mantel tests between temporal distance and genetic distance, and between spatial distance and either genetic distance or metabolic dissimilarity, all *p*-values > 0.05). Lastly, and not surprisingly, we found that genetic distance and metabolic dissimilarity were significantly positively correlated (Mantel test, $r = 0.188$ $p < 0.001$, Fig. 3). Taken together, these results suggest that temporally co-occurring strains may compete strongly for resources, a scenario that is thought to favour the evolution of antagonistic interactions (Schoustra *et al*., 2012, Cordero *et al*., 2012).

Is inhibition structured in space and time?

The spatial and temporal structure of inhibition amongst our isolates is shown schematically in figure 2, with isolates collected during the same growth season shaded grey. Consistent with the idea that strong resource competition can lead to antagonistic social interactions, we found that inhibition was significantly more likely to be observed between pairs of isolates collected within the same growing season than between growing seasons (Fig. 4A, binomial general linear mixed effect model on prevalence of inhibition with isolate identities as random effects: $p = 0.025$). A finer-scale analysis of the effect of month within year showed the same pattern (binomial general linear mixed model of the effect of temporal distance between isolates on the prevalence of inhibition, with sampling season and isolate identity as random effects, $p = 0.021$). Isolates were thus significantly more likely to exhibit

inhibition when they were sampled closer in time, on both large (sampling year) and small (sampling month) scales. In contrast, whether or not isolates were collected from the same transect did not impact the frequency of inhibition significantly (Fig. 4B, same model as for time but using transect instead of year: $p > 0.05$).

Is inhibition correlated with genetic or metabolic distance?

There is a tendency for the frequency of inhibition to decline with increasing genetic distance: inhibition was more likely when isolates had nearly identical 16S rRNA sequences (Fig. 4C), although this relationship was only marginally significant (general linear mixed model, effect of genetic distance on the prevalence of inhibition, isolate identities as random effects, $p = 0.055$). While metabolic dissimilarity seems to have a negative impact on inhibition prevalence, the model failed to converge (Figure 4D). Together, these results suggest that antagonistic interactions may play a role in resource competition, though we either lack statistical power or utilize too crude measures of diversity to detect a strong signal of this effect in our data set.

Discussion

The extent to which the immense diversity of bacterial communities can be attributed to the social interactions among strains or species remains unclear, in part because our understanding of the strength and frequency of these interactions outside the lab is limited. We have investigated the prevalence and specificity of antagonistic interactions among free-living pseudomonads, focusing on interactions mediated by bacteriocin-like exotoxins between 18 *Pseudomonas* isolates sampled from soil transects across a range of one kilometer over the course of two years. Within our set of strains inhibition was rare, with most isolates being resistant to exotoxins and only ~6% of interactions between pairs of isolates showing evidence of inhibition. When it was observed, exotoxin inhibition was more likely between pairs of strains collected closely together in time, and specifically from the same year of sampling. In contrast, isolates from the same transect were not more likely to inhibit each other than isolates sampled from a more distant transect. We found weakly negative relationships between exotoxin inhibition and genetic distance and the degree of resource overlap, such that related isolates utilizing the same resources show a tendency to

inhibit each other. Taken together, these results suggest that, while antagonistic interactions may be of some importance in resource competition, they do not appear to be a major agent of community structure of soil-dwelling *Pseudomonas*. We discuss these results and our interpretation of them in more detail below.

The frequency of exotoxin inhibition among free-living Pseudomonas strains

The low frequency of exotoxin inhibition in our collection is striking given the apparent ubiquity of exotoxins among bacteria (Riley & Gordon, 1999, Riley & Wertz, 2002), including *Pseudomonas* (Parret & De Mot, 2002, Ghoul *et al.*, 2015). This result does not, however, seem to be out of place among studies of antagonistic interactions in soil bacteria, including pseudomonads. Bruce and colleagues (2017), for example, detected a low prevalence of inhibition (8%) between soil-isolated *P. fluorescens* strains, and others have reported similarly low levels of antagonism in other bacteria (2-18%, Booth *et al.*, 1977, Davelos *et al*., 2004, Cordero *et al*., 2012, Perez-Gutierrez *et al.*, 2013). To be sure, such a low level of prevalence does not seem to be a definitive characteristic of soil bacteria. Other studies, for example, have documented much higher prevalence (from 38-51%) of antagonistic interactions among divergent bacterial groups isolated from soil (Vetsigian *et al*., 2004, Wilson *et al*., 2009, Hawlena *et al*., 2010*a*). Nor is it definitive to pseudomonads, as antagonistic interactions among *Pseudomonas aeruginosa* isolates derived from clinical settings have been observed to range from 50-80% (Schoustra *et al*., 2012, Ghoul *et al*., 2015) while those among rhizosphere *Pseudomonas ssp.* isolates were ~70% (Valdidov *et al*., 2005). The reasons for such vastly different frequencies of antagonism remain unclear at this point.

While it is tempting to speculate that these differences could be due to the different ecologies of the isolates tested if, for instance, isolates from high density environments such as hosts have higher rates of exotoxin production in order to mediate competition for resources, we cannot exclude methodological biases either. Our study, like many others (e.g. Long & Azam, 2001, Perez-Gutierrez *et al*., 2013, Ghoul *et al.*, 2015, Bruce *et al*., 2017), examined inhibition using cell-free extracts of producer strains grown in rich media. This approach could produce low levels of inhibition because it prevents the production of exotoxins that are expressed under starvation conditions and upon cell-to-cell contact or delivered via type III or type VI secretion systems. We may also have observed low

antagonism levels because we did not induce exotoxin production with heat or mytomycin C, as other studies have (Riley *et al.*, 2003, Hawlena *et al.*, 2012, Bruce *et al.*, 2017). Neither explanation is entirely satisfying, however, as there seems to be little evidence in the literature that such methods lead to consistent increases in the observed prevalence of anatagonism among natural isolates. For example, studies in pseudomonads have observed high levels of antagonism without induction (e.g. Schoustra *et al.*, 2012) and low levels of antagonism with it (Bruce *et al.*, 2017, 8% compared to 6% inhibitions in the present study), suggesting that induction is not directly tied to high rates of antagonism for a given sample. Thus there is little reason to supsect that our results are due exclusively to methodological biases. More broadly, the causes of variation in rates of antagonism among bacteria remain an important subject for future investigation.

Patterns of antagonism in time and space

Our observation that antagonism was more likely to occur between pairs of strains isolated closer in time but not in space is intriguing and warrants further investigation. We suspect that these patterns are connected to the strength of resource competition, as we found similar patterns for metabolic similarity, a proxy for resource use and thus niche overlap. Our observation of higher metabolic similarity of isolates sampled in temporal proximity is consistent with previous work indicating that bacterial environments change seasonally (Fuhrman *et al.*, 2006, Gilbert *et al.*, 2012, Kraemer & Kassen, 2015). It is also consistent with our previous work in this system that indicated that selection became more strongly divergent in time over two growing seasons while spatial variable selection did not (Kraemer and Kassen, 2015 & 2016). While it is tempting to interpret the turnover in bacterial genotypes over time as being driven by antagonistic interactions (e.g. Wilson *et al.*, 2009), the overall paucity of antagonism in our isolate collection suggests otherwise. Rather, we suspect the pattern arises because exotoxin-mediated inhibition tends to occur most strongly amongst ecologically very similar types (Hawlena *et al.*, 2012, Schoustra *et al.*, 2012) and that some other process — perhaps changing abiotic conditions of growth — generates a steady selective pressure leading to strain turnover at a given site (Szabo *et al.*, 2013).

The reasons underlying the absence of a spatial structure to antagonism in our study remain unclear. Most studies of antagonism focus on relatively fine spatial scales of a few millimeters or centimeters (Davelos *et al.*, 2004, Hawlena *et al.*, 2010*b*, Vetisgian *et al.*,

2011), suggesting that we may not have sampled at a sufficiently fine scale to detect antagonistic interactions. Two lines of evidence suggest this may not be the case. First, we have detected spatial variation in the strength of selection within a growing season in this system (Kraemer and Kassen, 2015), though not across different growing seasons (Kraemer and Kassen, 2016), and, second, others have detected antagonistic interactions across distances similar to the ones we have investigated (meters to kilometers; Hawlena *et al*., 2010*a*, Kinkel *et al*., 2014, Bruce *et al*., 2017). More information on the spatial scale of dispersal in this system, for example by utilizing trap cultures, would be useful to determine the scale at which antagonism is most likely to take place. Lastly, given the scarcity of antagonistic interactions in our sample, it may be that we simply lack the power to detect spatial structuring, if it exists.

Inhibition frequency as a function of ecological and genetic similarity

Antagonistic social interactions like exotoxin production are expected to evolve when resource competition occurs between closely related — though not identical — genotypes. The implication is that inhibition should be most prevalent between strains at intermediate levels of genetic divergence provided niche overlap declines with increasing genetic differentiation. As a consequence, there should be a hump-shaped relationship between the frequency of inhibition and genetic distance (Gardner *et al.*, 2004). While some studies have observed the expected humped-shaped relationship (Schoustra *et al.*, 2012, Cordero *et al.*, 2012), it is far from being a general result (Nair & Simidu, 1987, Hawlena *et al*., 2010*a*, Vetsigian *et al*., 2011, Cordero *et al*., 2012, Hawlena *et al*., 2012, Schoustra *et al*., 2012, Perez-Gutierrez *et al*., 2013, Bruce *et al*., 2017).

We similarily failed to detect a such a relationship. While metabolic dissimilarity increased with genetic distance, as required by the theory, we could not detect a humped-shaped relationship between genetic distance and the frequency of inhibition. If anything, our results suggest this relationship is negative, although again the low overall frequency of inhibition in our study leads to a lack of statistical power meaning that this result must be treated with caution. An alternative explanation is that our measure of genetic distance was too crude to be informative, being based on partial 16S rRNA sequences, which are typically used to resolve different species and genera of bacteria but not distinct genotypes within the same species, the likely targets for some subclasses of exotoxins such as bacteriocins (Lo

Giudice *et al*., 2007). Consistent with this interpretation, we detected two instances in which different isolates with a 16S rRNA genetic distance of zero inhibit each other, indicating some potential for exotoxins acting between closely related genotypes. Intriguingly though, a similar study of *P. fluorescens* isolates employing a more fine-scale genetic analysis also failed to find a relationship between antagonism and genetic distance, suggesting that toxin production and resistance might be correlated with only a few and fast evolving loci in the genome (Bruce *et al*., 2017). Future work is needed to describe the broader shape of the inhibition function across a range of genetic and metabolic distances.

Conclusion

Our study reveals that antagonistic interactions between soil pseudomonads are structured in time but not space. The low overall prevalence of inhibition does not lend support to the idea that exotoxin-mediated antagonistic interactions are a significant contributor to community structure for free-living soil *Pseudomonas*. Rather, we propose that these communities are structured by other selective factors that could involve more diffuse resource competition and the changing abiotic conditions of growth over the course of the growing season. Either way, exotoxin production seems to not represent a major selective force on soil pseudomonads on the scales investigated here.

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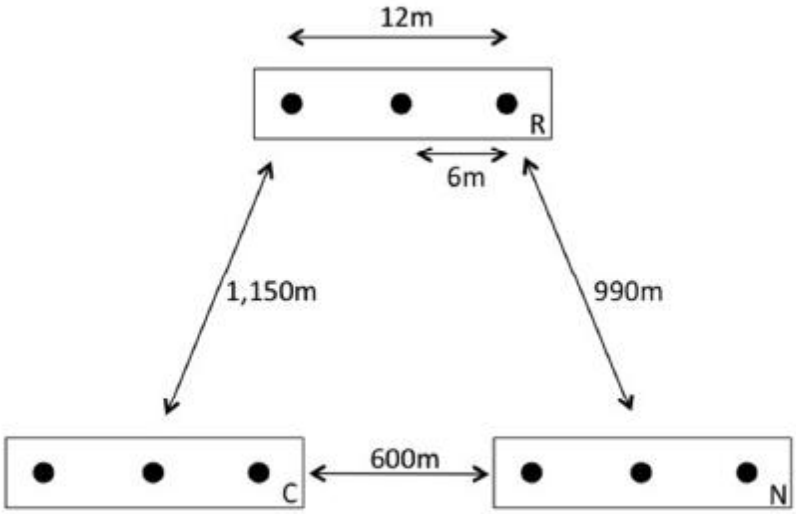

Figure 1: Schematic sampling map showing the three spatial sampling sites, as well as their distances from each other. Black dots represent meter-scale sampling points within each km-scale sampling site.

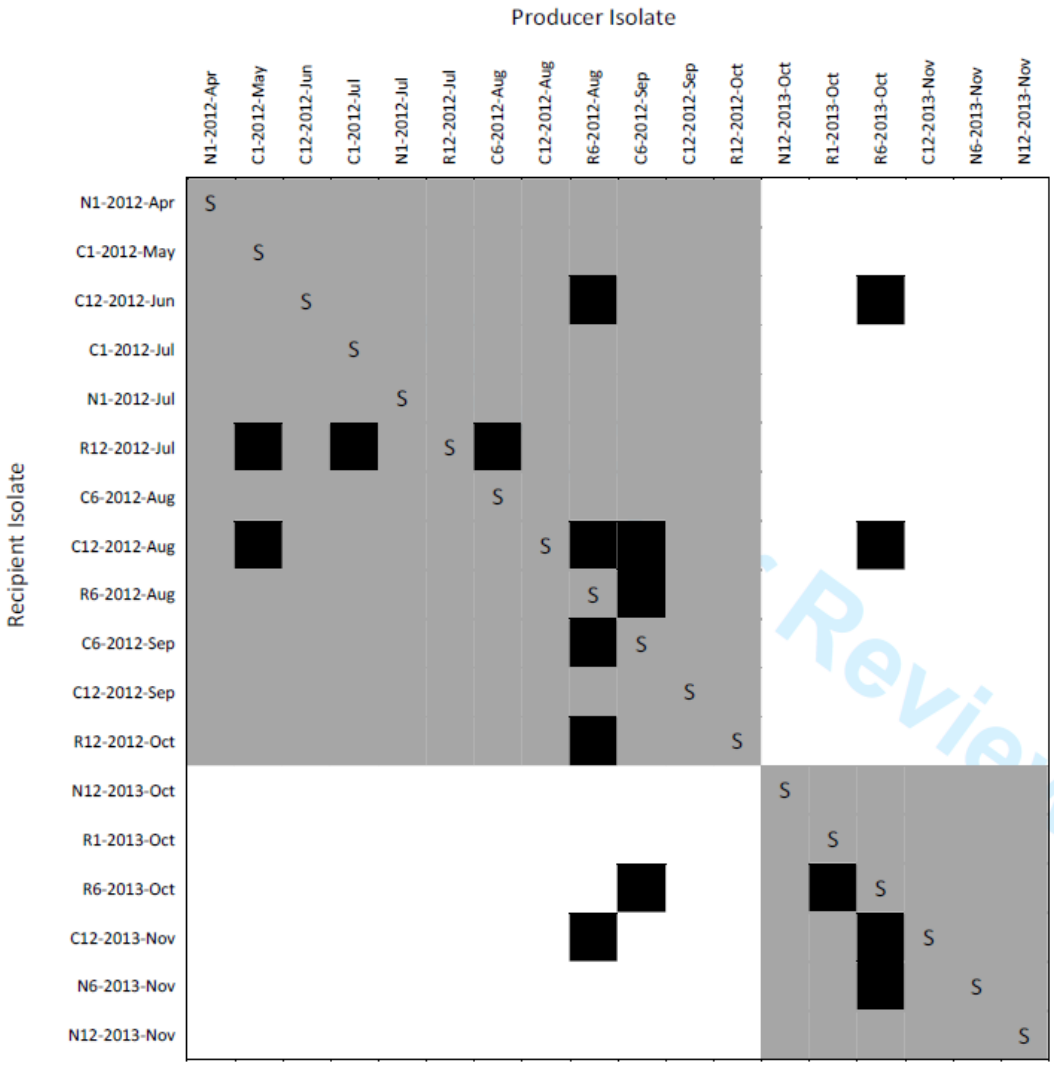

Figure 2: Exotoxin-mediated competition between 18 soil pseudomonads from the same (grey shading) or different (no shading) sampling years. Black fields indicate inhibitions, whereas empty fields indicate no observed inhibition of growth. 'S' indicates tests for self-inhibition.

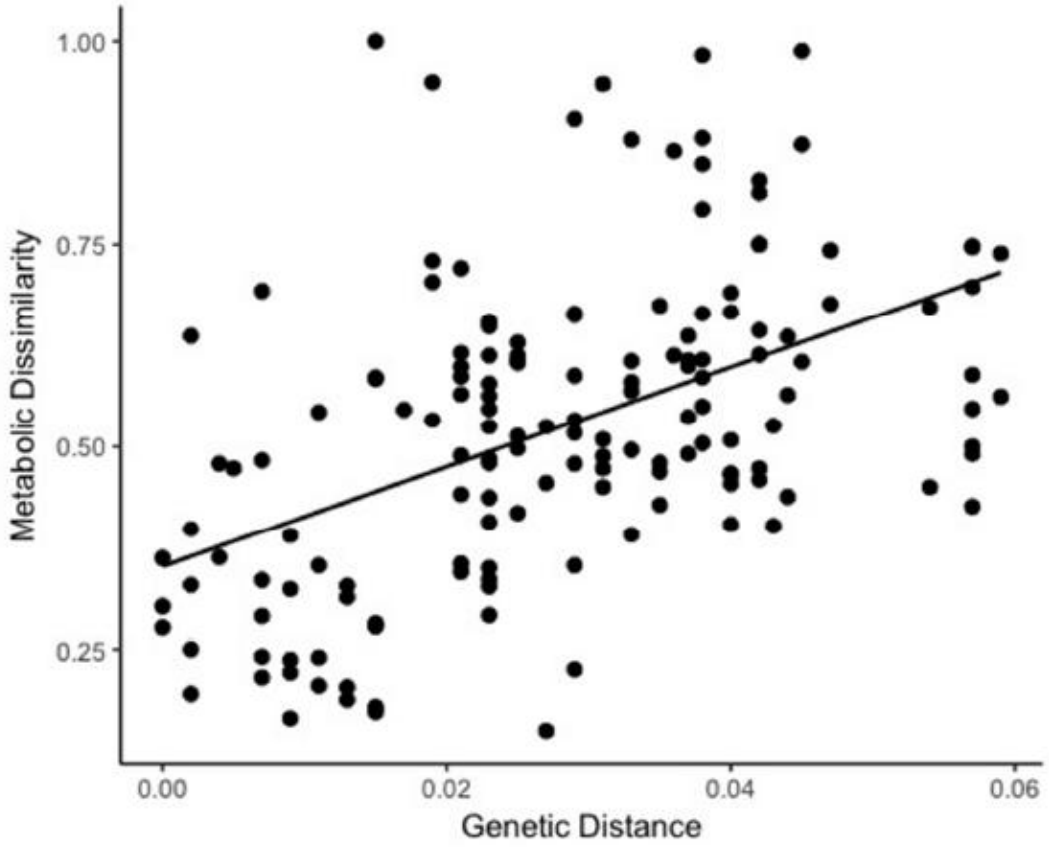

Figure 3: Correlation between metabolic dissimilarity and genetic distance based on the 16S rRNA gene of 18 soil pseudomonads (excluding self-comparisons).

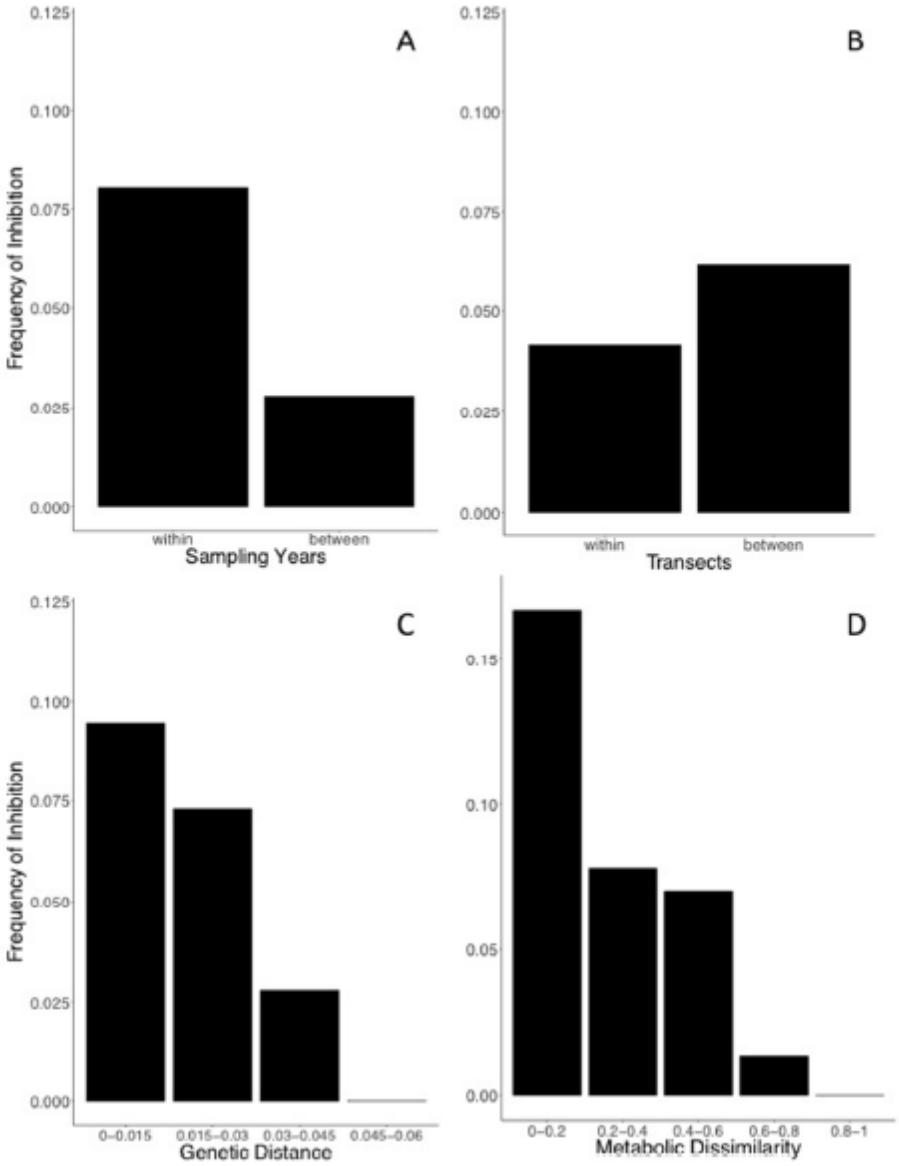

Figure 4: Frequency of inhibition between 18 soil pseudomonads (excluding self-comparisons, n = 306): A) within and between sampling years; B) within and between transects; C) across different classes of genetic relatedness; and D) across different classes of metabolic dissimilarity.