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The bacterium *Pseudomonas aeruginosa* senses and gradually responds to inter-specific competition for iron

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Abstract

Phenotypic plasticity in response to competition is a well-described phenomenon in higher organisms. Here, we show that also bacteria have the ability to sense the presence of competitors and mount fine-tuned responses to match prevailing levels of competition. In our experiments, we studied inter-specific competition for iron between the bacterium *Pseudomonas aeruginosa* (PA) and its competitor *Burkholderia cenocepacia* (BC). We focused on the ability of PA to phenotypically adjust the production of pyoverdine, an iron-scavenging siderophore. We found that PA up-regulates pyoverdine production early on during competition under condition of low iron availability. This plastic upregulation was fine-tuned in response to the level of competition imposed by BC, and seems to confer a relative fitness benefit to PA in the form of an earlier initiation of growth. At later time points, however, PA showed reduced growth in mixed compared to monoculture, suggesting that competitive responses are costly. Altogether, our results demonstrate that phenotypic plasticity in siderophore production plays an important role in inter-specific competition for iron. Upregulating siderophore production may be a powerful strategy to lock iron away from competing species, and to reserve this nutrient for strain members possessing the compatible receptor for uptake.

Keywords

inter-specific competition; phenotypic plasticity; microbe-microbe interactions; siderophores; competition sensing

Introduction

Under natural conditions, bacteria live in diverse communities, where competition for space and nutrients is intense (Whitman et al. 1998; Curtis et al. 2002; Becker et al. 2012; Foster

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Conflict of Interest

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and Bell 2012; Rinke et al. 2013). Bacterial traits, such as the secretion of toxins, the formation of protective biofilms, and the efficient scavenging of nutrients are usually interpreted as direct evolutionary adaptations to inter-specific competition (Weaver and Kolter 2004; Harrison et al. 2008; Bakkal et al. 2010; Tashiro et al. 2013; Oliveira et al. 2015). Less well understood, however, is to what extent bacteria can sense different levels of competition and adjust their phenotype accordingly. Because the expression of a ‘competitive phenotype’ is costly, it would make sense to solely express it when required, and to adjust its expression to match the prevailing level of competition. Phenotypic plasticity in response to competition is well documented for higher organisms (Agrawal 2001; Boege 2010; Leggett et al. 2014; Cortesi et al. 2015), and has recently also been put forward for bacteria (Cornforth and Foster 2013). Phenotypic plasticity, defined as the ability of a single genotype to express different phenotypes (West-Eberhard 1989), allows fast responses to environmental changes, which is thought to be adaptive, especially in fluctuating environments (Fordyce 2006).

Here, we use a laboratory model system to examine whether the bacterium *Pseudomonas aeruginosa* (PA) can sense and plastically respond to inter-specific competition for iron, when growing in co-culture with *Burkholderia cenocepacia* (BC). Iron is an essential nutrient and growth-limiting factor in most natural environments because ferric iron is insoluble at neutral pH or host-bound in the context of infections (Hersman et al. 2000; Miethke and Marahiel 2007). To overcome iron limitation, bacteria secrete siderophores, small secondary metabolites, which make ferric iron bio-available for all individuals with a specific receptor for uptake (Ratledge and Dover 2000; Miethke and Marahiel 2007; Tyrrell and Callaghan 2015). Previous research showed that siderophores play a crucial role for both intra- and inter-specific competition (Griffin et al. 2004; Weaver and Kolter 2004; West et al. 2006; Ross-Gillespie et al. 2007; Harrison et al. 2008; Kümmerli et al. 2009a; Traxler et al. 2013; Leinweber et al. 2017). In intra-specific competition, the secreted siderophores can be shared as public goods between cells that possess a compatible receptor for uptake. Sharing can select for cheating by mutants that no longer produce siderophores themselves, yet still capitalize on the siderophores produced by others (Griffin et al. 2004; West et al. 2006; Ross-Gillespie et al. 2007, 2015; Kümmerli et al. 2009a; Scholz and Greenberg 2015). For inter-specific competition, meanwhile, the secretion of efficient and exclusive siderophores, for which competitors do not possess receptors for uptake, seems to represent an efficient way to outpace the competing species in the race for iron (Weaver and Kolter 2004; Joshi et al. 2006; Harrison et al. 2008; Butait et al. 2017; Leinweber et al. 2017; Niehus et al. 2017; Schiessl et al. 2017).

To assess the role of siderophores for inter-specific competition, we carried out experiments in mixed-species communities, with PA as our focal strain and BC as its competitor. Both species rely on a primary siderophore for iron scavenging that cannot be cross-used between species. These are pyoverdine for PA (Ankenbauer et al. 1985) and ornibactin for BC (Mathew et al. 2014). Our previous work showed that pyoverdine is superior over ornibactin and plays an important role in the competition between the two species (Leinweber et al. 2017). In addition, PA and BC produce the same secondary siderophore pyochelin. Since pyochelin is predominantly produced when iron availability is increased, it should not influence inter-specific competition under low iron availability, but might do so when iron is

more readily available (Dumas et al. 2013; Tyrell et al. 2015). PA possesses sophisticated regulatory mechanisms to fine-tune pyoverdine synthesis in response to prevailing levels of iron limitation (Leoni et al. 1996; Lamont et al. 2002; Tiburzi et al. 2008). When intracellular iron concentration is high, ferrous (Fe^{2+}) iron forms a complex with Fur (ferric uptake regulator), which in turn suppresses the expression of *pvdS* (the gene encoding the iron starvation sigma factor). Conversely, if iron is scarce, *pvdS* is expressed and triggers the transcriptions of genes involved in pyoverdine synthesis via a non-ribosomal peptide synthesis assembly line (Visca et al. 2007). It was shown that the interplay between these negative and positive feedback loops allows fine-tuned response to changes in both the abiotic and biotic environment (Kümmerli et al. 2009b).

We used this study system to address the following questions. First, we asked whether PA generally adjusts the production of pyoverdine in response to inter-specific iron competition. To tackle this question, we grew PA in mono- and mixed culture with BC in three different environments varying in their level of iron limitation, and quantified the variation in PA's pyoverdine production profiles. Second, we asked whether phenotypic plasticity in response to competition involves a binary switch from one phenotype to another (i.e. on/off competition stage) or whether responses can be more gradually adjusted to match the prevailing level of competition. To answer this question, we exposed PA to various levels of BC competition by modifying the mixing ratio of the two species. Since BC is a relatively weak competitor (Leinweber et al. 2017), we hypothesized that PA should primarily adjust its pyoverdine phenotype when competitive pressure from BC is high. Third, we explored whether phenotype adjustments in pyoverdine production have fitness consequences for PA. This is a crucial point because if competition sensing and the resulting change in phenotype were adaptive then adjustment of siderophore phenotypes should entail fitness benefits. Finally, we examined whether pyochelin affects pyoverdine production profiles of PA in mono and mixed cultures, and whether this secondary siderophore influences inter-specific competition.

Methods

Bacterial Strains

For our main experiments, we used *P. aeruginosa* strain PAO1 (ATCC 15692) and *B. cenocepacia* strain H111 (LMG 23991), an isolate from a cystic fibrosis patient (Gotschlich et al. 2001). To be able to distinguish PA from BC in mixed cultures, we used PAO1-*mcherry*, a variant of PA that constitutively expresses the fluorescent protein mCherry (chromosomal insertion *attTn7::Ptac-mcherry*) for all experiments. H111 did not carry a fluorescent marker. For some experiments, we further used the reporter strain PAO1*pvdA-gfp*, where *gfp* expression is under the control of the *pvdA*-promoter (chromosomal insertion *attB::pvdA-gfp*) (Kaneko et al. 2007), and which also featured a constitutively expressed mCherry marker (chromosomal insertion *attTn7::Ptac-mcherry*). Since PvdA catalyzes a key step in the production of pyoverdine (Visca et al. 1994; Leoni et al. 1996), the expression level of *pvdA* serves as a proxy for the investment into the pyoverdine assembly line, and thus pyoverdine production (Weigert and Kümmerli 2017).

Finally, we used a number of additional strains for control experiments. To assess whether pyochelin affects the competition between PAO1 and H111, we repeated parts of our main experiments with strains deficient for pyochelin production. These strains were PAO1 *pchEF* (Ghysels et al. 2004) and H111 *pchAB* (Mathew et al. 2014). Furthermore, we used H111-*mcherry* (chromosomal insertion *attTn7::Ptac-mcherry*), a BC strain constitutively expressing mCherry to quantify the growth of BC in mixed culture with PA.

Media and Growth Conditions

Overnight cultures were grown in lysogeny broth (LB). All experiments were conducted in casamino acids (CAA) medium (per 1 liter: 5 g casamino acids; 1.18 g $K_2HPO_4 \cdot 3H_2O$; 0.25 g $MgSO_4 \cdot 7H_2O$) supplemented with 20 mM $NaHCO_3$ and 25 mM HEPES buffer. We induced strong iron limitation by adding 100 $\mu g/ml$ of the natural iron chelator human apo-transferrin. Under these conditions, strains without siderophores cannot grow (Kümmerli et al. 2009b), and thus we expect strong competition for iron between PA and BC. Intermediate iron levels were achieved by adding 0.5 μM $FeCl_3$ to CAA medium with 100 $\mu g/ml$ human apo-Transferrin. Previous results have shown that siderophores are still important for growth, but PA halves pyoverdine production under these conditions (Kümmerli et al. 2009b). To create iron-replete medium, we added 20 μM $FeCl_3$ and omitted transferrin from the above recipe. With such high levels of iron supplementation, siderophore production is strongly suppressed and transferrin no longer limits iron availability (Dumas et al. 2013). We assume that there is weak competition for iron between PA and BC under these conditions. All chemicals were purchased from Sigma-Aldrich, Switzerland.

Measuring Pyoverdine Production Profiles in Response to Competition

We designed a multi-factorial experiment to test whether PA can sense inter-specific competition for iron and adjust its pyoverdine production accordingly. The first factor compared the pyoverdine production profiles of PA when grown in mono- versus mixed culture with BC. The second factor focused on the density of PA, thereby manipulating the level of competition between the two species in mixed cultures. Here, we asked whether PA gradually adjusts its pyoverdine production phenotype when exposed to higher densities of BC (i.e. higher levels of competition). The third factor examined the effect of iron availability on pyoverdine production profiles. The prediction here is that phenotypic plasticity in response to iron competition should mainly manifest at low iron availability, when competition for this nutrient is most intense.

Prior to the experiment, PA and BC were grown overnight in LB medium, harvested by centrifugation (7500 rpm, 2 min), and washed twice with 0.8 % NaCl solution. Cultures were then adjusted to $OD_{600} = 1$ (optical density at 600 nm) in 0.8 % NaCl. Subsequently, we mixed PA and BC in different volumetric ratios (0.5 % to 99.5 %; 1 % to 99 %; 5 % to 95 %; 10 % to 90 %; 50 % to 50 %, respectively) for the inter-specific competition treatments. This procedure keeps the overall starting cell density constant, whilst varying the strength of competition PA faces (i.e. strongest and weakest level of inter-specific competition occur at 0.5 % to 99.5 % and 50 % to 50 %, respectively). For the PA monocultures, we chose the same initial PA cell densities, and mixed these cultures with 0.8 % NaCl without BC, using the same volumetric ratios as indicated above. A total volume

of 20 μ l of these bacterial cultures were then inoculated into CAA medium (featuring the three iron concentrations described above) in sterile 2 ml reaction tubes (Sarstedt) to an initial OD₆₀₀ of 1×10^{-2} . This mixing procedure allowed us to keep the NaCl-induced dilution factor constant. We then distributed the inoculated medium to 96-well plates (200 μ l per well) in 7 to 8 replicates per treatment, and incubated the cultures in a Tecan Infinite M 200 plate reader (Tecan Group Ltd., Switzerland) at 37°C. To quantify pyoverdine levels, we made use of the auto-fluorescence properties of pyoverdine, which can be excited at 400 nm, and fluorescence measured at 460 nm (Ankenbauer et al. 1985). Pyoverdine fluorescence was measured every 15 min for 48 h. Prior to each reading event, plates were shaken for 30 seconds (3.5 mm orbital displacement).

To analyze the pyoverdine production profiles, we fitted non-parametric spline models to the fluorescence data, using the *grofit* R package (Kahm et al. 2010). We used spline fitting because pyoverdine trajectories followed non-logistic patterns and varied greatly between the different treatments (Figure S1). We took the integral of the spline fits (i.e. area under the curve) as a proxy for the cumulative pyoverdine availability over time, and for comparisons of the global pyoverdine production profiles between the different treatments (i.e. mono vs. mixed culture, level of competition, iron concentration). To obtain differences in the temporal dynamics of pyoverdine production between mono and mixed cultures, we calculated the mean difference of pyoverdine fluorescence across replicates for each time point and treatment as: *mean pyoverdine fluorescence of mixed culture – mean fluorescence of the respective monoculture*. To estimate standard errors of this concatenated variable, we applied the *jackknife* function from the *bootstrap* R package (Tibshirani and Leisch 2015).

Measuring Growth Trajectories in Mono- and Mixed Cultures

To follow PA growth trajectories in mixed and monocultures, we used the same experimental setup described above. Since optical density (the standard measure for growth) cannot be used to follow PA growth in mixed cultures with BC, we relied on the constitutively expressed mCherry marker to track PA growth. We measured the mCherry fluorescence (excitation = 582 nm, emission = 620 nm) of PA every 15 min for 48 h with the plate reader under the same culturing conditions as described above.

We carried out two control experiments to check whether mCherry is a good proxy for growth. First, we grew PAO1-*mcherry* as monoculture, and simultaneously measured OD₆₀₀ and mCherry fluorescence for all experimental conditions described above. We then superimposed the two readouts. We found that the trajectories paralleled decently for OD and mCherry signals during the early and the exponential growth phase (Figure S2). In contrast, the correlation between the two signals broke down when cultures reached stationary phase (according to OD), as growth stops but cells continue to produce and accumulate mCherry. For this reason, we only extracted growth parameters during the early and exponential growth phase for fitness comparisons. For these we found significant positive correlations (Figure S3). Second, we checked whether mCherry fluorescence is influenced by the presence of BC. For this test, we took undiluted overnight cultures of PA (PAO1-*mcherry*) and BC, washed them and adjusted their OD to 1. We then diluted the PA culture in multiple steps (100 %, 50 %, 25 %, 12.5 %, 6.25 %, 3.125 %, 0 %) in either 0.8 %

NaCl buffer or the BC culture. We then directly measured mCherry fluorescence in mixed cultures and monocultures. We found strong linear correlations between PA density and mCherry fluorescence, with the patterns not being influenced by the presence of BC (Figure S4).

Having established that mCherry is a reliable measure for growth, we again used the grofit package (Kahm et al. 2010) to obtain quantitative estimates of growth parameters from the observed mCherry fluorescence trajectories (Figure S5). The package allows to fit multiple parametric growth models, and uses the AIC (Akaike Information Criterion) to identify the best fits. Across all our analyses, the Gompertz models yielded the most accurate fits to the data. From the model fits, we extracted: (a) the duration of the initial phase (IP), which is defined as the part of the lag-period, where growth rate equals zero (Prats et al. 2008); and (b) the maximum growth rate μ , which is the maximal slope of the growth curve. Figure S6 shows a number of curve fits for illustration.

Single Cell *pvdA* Gene Expression Analysis

The above experiments will allow us to establish correlations between pyoverdine levels and growth trajectories. However, the actual causality between the two variables will remain concealed. For example, it is possible that: (i) a higher pyoverdine production level per cell leads to accelerated growth; or (ii) cells proliferate faster due to other reasons, which would result in higher pyoverdine availabilities at the population level without individual cells actually changing their pyoverdine production. To disentangle these two options, we directly quantified the expression of *pvdA* in PA at the cellular level using fluorescence microscopy. To prepare microscopy samples, we first grew monocultures of the PA reporter strain (PAO1 *pvdA-gfp mcherry*) and BC in LB overnight and subsequently harvested cells by centrifugation at 7500rpm, followed by two washing steps in 0.8 % NaCl. The cultures were adjusted to $OD_{600} = 1$ and mixed in a ratio of 0.5 % PA to 99.5 % BC. For PA monocultures, we added the same volume of 0.8 % NaCl without BC. Iron limited CAA medium (supplemented with 100 μ g/ml transferrin) was then inoculated with PA only or a mixture of PA and BC, in four 96-well plates (200 μ l per well) to a starting OD_{600} of 1×10^{-2} . As a control where no *pvdA* expression is expected, we inoculated PA as monoculture in iron replete CAA medium supplemented with 100 μ M $FeCl_3$. The four plates were incubated at 37°C and 170 rpm in an orbital shaker. At each of the four time points (10, 12, 14 and 20 hours after inoculation), we processed one of the 96-well plates for microscopy. Specifically, we diluted the grown cultures in 0.8 % NaCl to appropriate levels and then added 1 μ l of the culture onto a 1 % agarose pad (only containing 0.8% NaCl) on a standard microscopy slide. Agarose pad preparation followed the protocol described in Weigert and Kümmerli (2017).

For all the mCherry positive cells (expressed by the PA reporter strain only), we measured the *pvdA* associated GFP fluorescence. Imaging was performed with a widefield Leica DMI6000 microscope equipped with a plan APO PH3 objective (NA = 1.3), an automated stage and a Leica DFC 350 FX camera (resolution: 1392x1040 pixels) for image recording (16 bit color depth). We used a Leica L5 filter cube for GFP (emission: 480 ± 40 nm, excitation: 527 ± 30 nm, DM = 505) and a Leica TX2 filter cube for mCherry (emission: 560 ± 40 nm, excitation: 645 ± 75 nm, DM = 595) fluorescence. The exposure time for

measuring GFP-fluorescence was 268 ms and for mCherry 182 ms with a (halogen) lamp intensity of 100%. We analyzed between 144 and 642 mCherry-positive PA cells per treatment and time point. Slide preparation followed by imaging took approximately an hour, a timeframe during which GFP remains stable, such that we can be confident that the fluorescent level measured per cell reflects their gene expression during the experiment. Single cell analysis followed the method described in Weigert and Kümmerli (2017). For image segmentation we used the supervised object classification and segmentation tool ilastik (Sommer et al. 2011). Images were then further analyzed with Fiji, a free scientific image processing software package (Schindelin et al. 2012).

Statistical Analysis

All statistical analyses and model fitting was carried out in R 3.1.2 (R Development Core Team 2015).

We used analysis of covariance (ANCOVA) to test for significant effects of the three main variables and their interactions. Specifically, we included the degree of iron limitation and the absence/presence of the competitor BC as categorical variables, and the level of competition (Figure 1 and 3) or time (Figure 4) as covariates into our models. While we worked with complete models for the analysis of the overall pyoverdine production (Figure 1) and *pvdA* expression over time (Figure 4), we constructed separate models for each growth parameter and degree of iron limitation for the fitness analysis (Figure 3). We applied two-sample *t*-tests, to analyze whether pyoverdine production profiles differ between mono- and mixed cultures at specific mixing ratios (Figure 1), or whether *pvdA* expression differs at specific time points (Figure 4). We used the Shapiro-Wilk-Test to check for normal distribution of the model residuals, prior to all analyses.

Results

Iron, the Presence of Competitors, and the Level of Competition Affect Pyoverdine Production

When analyzing the overall pyoverdine profiles across 48 hours, we found that pyoverdine levels significantly varied in response to iron concentration, the absence/presence of a competitor, and the level of competition between PA and BC (Figure 1). Consistent with previous studies (Kümmerli et al. 2009b; Dumas et al. 2013), our data show that PA gradually increases pyoverdine production when iron becomes more restricted (Linear model (LM): $F_{2, 220} = 5702.3$, $p < 0.0001$; Figure 1). Moreover, we found that the presence of the competitor BC significantly reduced global pyoverdine levels in mixed compared to PA monocultures, with the effect being significantly more pronounced with stronger levels of competition (LM: significant interaction between the initial density of PA and the competition treatment (mixed vs. monoculture), $F_{1, 220} = 22.4$, $p < 0.0001$; Figure 1).

Differences in Temporal Pyoverdine Levels in Response to Competition

Next, we focused on the temporal variations in pyoverdine levels. While Figure S1 shows the raw pyoverdine trajectories for all treatments, we here analyze the differences between mixed cultures (where PA grew with BC) and monocultures (Figure 2). These analyses

revealed higher pyoverdine levels in mixed compared to monocultures during the early competition phase (i.e. up to 18 hours), but only under low iron availability (positive peaks in Figure 2a). The amplitude of the pyoverdine peaks (i.e. higher levels of pyoverdine in mixed versus monocultures) gradually declined with decreasing levels of competition. While small pyoverdine peaks were also detectable at intermediate iron availability (Figure 2b), they completely disappeared under iron-replete conditions (Figure 2c). Overall, the positive peaks indicate that PA upregulates pyoverdine production when encountering an inter-specific competitor under low iron availability, and fine-tunes this upregulation in response to the level of competition (i.e. the density of competitors).

We further observed time windows where the levels of pyoverdine were lower in mixed versus monocultures (Figure 2). Since these negative peaks appeared at all three levels of iron availability, and coincided with reduced PA growth in mixed compared to monocultures during the exponential growth phase (Figure S5), the patterns seem to be unlinked to iron competition. The amplitude of these negative peaks diminished with decreasing levels of competition (i.e. the density of competitors), and appeared at earlier time points when iron was supplemented, which is attributable to the accelerated growth observed with higher iron availabilities (Figure S5).

Temporal Differences in Pyoverdine Levels Correlate with Fitness Parameters

We next analyzed the fitness consequences of our treatments for PA (Figure 3). Under low iron availability, we found that the initial phase (IP, where growth rate equals zero) is significantly shorter in mixed compared to monocultures with the effect being most prominent under high levels of competition (LM: significant interaction between culture treatment (mono vs. mixed) and initial PA density, $F_{1,76} = 146.8$, $p < 0.0001$; Figure 3a). The same pattern was also apparent at intermediate iron availabilities ($F_{1,75} = 4.3$, $p = 0.041$; Figure 3b) but disappeared under high iron availabilities ($F_{1,66} = 1.8$, $p = 0.19$; Figure 3c). These data show that the increased availability of pyoverdine early during competition with BC in iron-limited media (Figure 2a) correlates with an earlier initiation of PA growth in mixed compared to monocultures.

This fitness advantage during the initial growth phase turned into a disadvantage during later stages of competition, where the growth rate of PA was reduced in mixed compared to monocultures (Figure 3d-f). In all iron treatments, fitness values between mixed and monocultures differed most clearly when competition for PA was strong (i.e. low density of PA relative to BC), but converged when the level of competition was reduced (i.e. high density of PA relative to BC; significant interactions between culture treatment (mono vs. mixed) and initial PA density; LM for low iron availability: $F_{1,76} = 10.1$, $p = 0.002$; intermediate iron availability: $F_{1,75} = 240.8$, $p < 0.0001$; high iron availability: $F_{1,66} = 32.2$, $p < 0.0001$). These data show that the decreased availability of pyoverdine during the exponential growth phase in competition with BC could stem from the reduced growth rates of PA in mixed compared to monocultures.

PA Actively Upregulates Pyoverdine Synthesis in Response to Competition

There are two causal explanations for the strong correlations observed between the changes in pyoverdine profiles and fitness patterns in response to competition. First, PA could upregulate pyoverdine production in response to strong inter-specific iron competition, which in turn stimulates a faster initiation of growth. Alternatively, PA might start proliferating faster in response to competition. Faster growth would then lead to higher cell numbers and thus to more pyoverdine being available at the population level, without individuals themselves changing their pyoverdine production rate. In order to disentangle these two scenarios, we examined the expression of the *pvdA* gene at the single-cell level. *PvdA* encodes an enzyme that is involved in pyoverdine synthesis via a non-ribosomal peptide synthesis assembly line (Visca et al. 1994; Leoni et al. 1996). We found that *pvdA* expression was significantly increased in mixed compared to PA monocultures early on during the competition (Figure 4; *t*-test for difference between mixed and monoculture in iron limited medium at 10h after inoculation: $t_{480} = 12.86$, $p < 0.0001$; after 12h: $t_{269} = 6.52$, $p < 0.0001$; after 14h: $t_{782} = 12.27$, $p < 0.0001$). The pattern then flipped later on during the competition, where *pvdA* expression was significantly reduced in mixed compared to monocultures (Figure 4; *t*-test at 20h after inoculation: $t_{325} = -6.7$, $p < 0.0001$). In both treatments, we observed an overall increase in the fluorescence signal over time, which is likely attributable to the fact that GFP is relatively long-lived and thus accumulates within cells. Taken together, our single-cell gene expression analysis provide support for the first causal explanation: inter-specific competition for iron stimulates increased pyoverdine synthesis during the early stages of competition under conditions where siderophores are essential for iron scavenging, which results in a fitness advantage for PA, in the form of an earlier onset of growth (Figure 3a).

Growth of BC is Strongly Reduced in Co-Culture with PA

Another important point is to understand what happened to BC during competition with PA, and whether the growth rate reduction PA experiences in mixed culture is due to BC growing and consuming resources. To address this issue, we tracked the growth of BC in mixed and monoculture (Figure S7), and assessed its relative fitness compared to PA (Figure S8). We found that the growth of BC was strongly repressed in the presence of PA (Figure S7), and BC was significantly outcompeted under all iron treatments (Figure S8). While BC did not show detectable growth when mixed at a 1:1 ratio with PA, there was low but detectable growth when BC was more abundant (99.5 %). Given the poor growth of BC, we argue that the PA growth rate reduction is unlikely explained by BC consuming significant amounts of resources.

Pyochelin is Involved in the Growth Rate Reduction Experienced by PA

As mentioned above, PA and BC can both produce the secondary siderophore pyochelin, which has lower affinity for iron than pyoverdine or ornibactin (Ankenbauer et al. 1985; Dumas et al. 2013; Mathew et al. 2014; Tyrell et al. 2015). Nonetheless, there is the potential that the two species can use each other's pyochelin for iron scavenging, and that some of the pyoverdine profiles and fitness patterns observed here are influenced by pyochelin. To check this possibility, we repeated our main experiments with PA and BC strains that are unable to

produce pyochelin (i.e. due to deletions in synthesis genes). For these experiments, we only considered the two most extreme mixing ratios between PA and BC (0.5 % : 99.5 % corresponding to high levels of competition for PA; and 50 % : 50 % corresponding to low levels of competition for PA). It turned out that no matter whether we analyzed the global pyoverdine profiles across 48 hours (Figure S9) or the temporal differences in pyoverdine levels between mixed and monocultures (Figure S10), we recovered the same qualitative patterns for the pyochelin-deficient and the wildtype PA strains. This suggests that pyochelin is neither responsible for the early up-regulation of pyoverdine (positive peaks in Figure S10) under conditions of low iron availability and high levels of competition with BC, nor for the lower pyoverdine levels (negative peaks in Figure S10) observed later on during competition.

We then analyzed the fitness parameters (initial phase IP, and growth rate) for the pyochelin-deficient PA strain and compared them to the PA wildtype strain (Figure 5). Under low iron availability, we recovered the previous results that IP was significantly shorter in mixed compared to monoculture environments, with the effect being most prominent with high levels of competition (LM: significant interaction between culture treatment (mono vs. mixed) and initial PA density, $F_{1,16} = 75.3$, $p < 0.0001$; Figure 5a). This pattern was still apparent at intermediate iron availability ($F_{1,16} = 28.4$, $p < 0.0001$; Figure 5b), but disappeared at high iron availability ($F_{1,16} = 4.3$, $p = 0.054$; Figure 5c). Thus, pyochelin does not seem to be involved in the earlier onset of growth observed in mixed compared to monocultures under low iron availability.

In contrast, the ability to produce pyochelin seems to play a role in the reduction of the PA growth rate observed in mixed versus monocultures later on during competition. Specifically, in competitions between the pyochelin-deficient PA and BC strains, we recovered the observed growth rate reduction for PA only at low iron availability ($F_{1,16} = 17.7$, $p < 0.001$; Figure 5d), but no longer at intermediate ($F_{1,16} = 0.1$, $p = 0.818$; Figure 5e) and high ($F_{1,16} = 1.2$, $p = 0.291$; Figure 5f) iron availabilities. Moreover, we found that the PA growth rate was overall significantly reduced in the pyochelin-deficient PA strain compared to the PA wildtype strain under intermediate ($F_{1,32} = 1645.5$, $p < 0.0001$; Figure 5e) and high ($F_{1,32} = 4458$, $p < 0.0001$; Figure 5f) iron availabilities, suggesting that pyochelin is important for iron-scavenging under those conditions.

Discussion

It has long been recognized that siderophores are important modulators of intra-specific competition, as mutants that are defective for siderophore synthesis can exploit the siderophores produced by others, thereby compromising the fitness of producers and the community as a whole (Griffin et al. 2004; West et al. 2006; Ross-Gillespie et al. 2007, 2015; Kümmerli et al. 2009a; Scholz and Greenberg 2015). Here, we show that siderophores are also important factors in inter-specific competition. In particular, we found that: (a) the bacterium *P. aeruginosa* (PA) up-regulates the production of its primary siderophore, pyoverdine, during the early phase of competition against *B. cenocepacia* (BC) under low iron availability; (b) this phenotypic plastic upregulation was fine-tuned in response to the level of competition imposed by BC; and (c) the early upregulation of pyoverdine confers a

fitness benefit in the form of an earlier initiation of growth in mixed compared to monocultures. Our findings suggest that PA cannot only sense the presence of BC, but also the level of competition imposed by this competitor, and gradually adjust its pyoverdine production to match prevailing conditions. Our results indicate that siderophores also play an important role in inter-specific competition by accessing and binding iron before the competitor does so (Fgaier and Eberl 2010, 2011; Leinweber et al. 2017; Niehus et al. 2017; Schiessl et al. 2017). This strategy could lock iron away from the competitor, and reserve it for strain members possessing the matching receptor for uptake – a strategy that seems particularly successful when siderophore production is increased in response to competition. More generally, our findings indicate that phenotypic plasticity in response to competition is not restricted to higher organisms, but might also be a common feature in microorganisms.

We can offer three mutually non-exclusive explanations for how PA could sense the level of inter-specific competition for iron in such a fine-tuned manner under low iron availability. First, PA might sense the presence of BC via indirect cues, such as increased resource competition. For instance, a realistic scenario under low iron availability is that BC secretes its own siderophore ornibactin (Tyrrell et al. 2015), which binds iron and thereby reduces its availability for PA. The early upregulation of pyoverdine by PA could then be understood as a response to increased iron limitation (Weaver and Kolter 2004). As mentioned in the introduction, fine-tuned regulation in response to competition could operate through interactions between the negative Fur feedback, curbing production levels when intra-cellular iron stocks become replenished (Leoni et al. 1996), and the positive feedback mediated by pyoverdine-signaling, which triggers increased pyoverdine synthesis (Lamont et al. 2002; Tiburzi et al. 2008). Second, PA could directly sense diffusible molecules secreted by BC and use those as cues to mount a specific response. Candidates for such diffusible cues are quorum sensing molecules, extra-cellular enzymes, or toxins (Jimenez et al. 2012). These molecules could be detected via specific receptors (i.e. a phenomenon called ‘cross-talk’ for quorum sensing molecules (Williams 2007)) or through their negative effects. For example, diffusible molecules such as extra-cellular enzymes or toxins can induce cell wall damage, DNA damage, or oxidative stress (Cornforth and Foster 2013), which could trigger responses as those observed in our study. Finally, competition sensing could also occur through direct cell-to-cell contact, whereby structural elements of the cell envelop could serve as cues to mount responses (Hood et al. 2010; Silverman et al. 2012; Basler et al. 2013; Ruhe et al. 2013; Mercy et al. 2016; McNally et al. 2017).

We might ask why PA does not respond to competition when mixed at a 1:1 ratio with BC? Previous work and our results suggest that this is because PA is a very strong competitor, simply outgrowing BC in many cases (Figures S7+S8; Leinweber et al. 2017). Thus, it seems that no specific competitive responses from PA against BC are required at an equal strain ratio, as PA is certain to win anyway. Conversely, BC might impose a more serious threat to PA when initially dominant (90 % in the population), and this seems to trigger beneficial competitive responses as the one observed for pyoverdine. Given these insights, we would expect that whether or not competitive responses are mounted will depend on the strength of the opponent, with the induction of responses presumably occurring at higher strain frequencies the stronger the opponent is.

While we postulate that the early up-regulation of pyoverdine by PA in competition with BC is a beneficial response as it spurs an earlier initiation of growth under low iron availability, the significant reduction in growth rate experienced by PA in mixed culture with BC is more difficult to explain (Figure 3d-f). We assume that the drop in growth rate reflects a general response to competition because it occurred across all levels of iron availability, and the magnitude of the response declined with lower levels of competition. But what could be the underlying causes for the growth rate drop? One obvious explanation is that BC consumes nutrients, thereby slowing down PA growth. Our control experiments revealed that BC manages to grow, although poorly, when seeded at high initial frequency into the co-culture (Figure S7). However, we believe that the marginal BC growth observed is unlikely to explain the marked drop in growth rate because PA reduced growth long before nutrients became exhausted (compare Figures 3d-f with S4). Instead, data from our experiments with pyochelin-negative mutants suggest that this secondary siderophore plays a role in the PA growth rate drop, especially under condition of intermediate and high iron availability (Figures 5e+f). Previous work showed that PA gradually changes its siderophore portfolio from pyoverdine to pyochelin when iron becomes more readily available (Dumas et al. 2013). In competition against BC, this means that PA changes from producing the exclusive pyoverdine to producing the shareable pyochelin, which can potentially be exploited by BC, and thus reduce PA growth. Additionally, a general drop in growth rate could also be explained by the fact that PA is likely to react at multiple fronts to inter-specific competition. For example, PA has been shown to increase biofilm formation, pyocyanin production and to activate the type-6-secretion system when facing a competitor (Basler et al. 2013; Trejo-Hernandez et al. 2014; LeRoux et al. 2015; Oliveira et al. 2015). The expression of this arsenal likely incurs significant metabolic costs, which are expected to impair growth rate, as observed in our experiments (Figure 3d-f).

In conclusion, our study reveals that PA plastically adjusts the synthesis of its primary siderophore in response to the level of competition with BC, a response that had direct consequences for PA's fitness. Our study thus highlights that siderophores are important agents in inter-specific competition for iron. This contrasts with their role as growth-promoting public goods, which can be cooperatively shared between members of the same strain. Both within-species cooperation and between-species competition seem to be important elements contributing to community assembly and composition. Accordingly, integrative studies focusing on both types of interactions in multi-species communities are required to better understand the relative importance of the two roles of siderophores.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Overall pyoverdine levels in *P. aeruginosa* (PA) cultures are influenced by inter-specific competition with *B. cenocepacia* (BC) and iron availability in the medium. High levels of competition with BC reduce overall pyoverdine levels compared to the respective PA monocultures, whereas pyoverdine levels converge in mixed compared to monocultures when competition levels decline. Across all levels of competition, pyoverdine levels increase when iron availability decreases. In mixed and monocultures, we first manipulated the density of PA (expressed as percentage of OD₆₀₀ = 0.01). For mixed cultures, we subsequently manipulated the level of competition by adding different amounts of BC (highest level of competition: 0.5 % PA vs. 99.5% BC; lowest level of competition 50 % PA vs. 50 % BC). In monocultures, we added respective amounts of NaCl buffer instead of BC. Bacterial cultures were grown in CAA media under low (blue), intermediate (red) and high (orange) iron availability. Circles depict individual data points (7 to 8 replicates per treatment) and values represent the integral of the global pyoverdine fluorescence profile (area under the curve across 48 hours, Figure S1). Black symbols depict means $\pm$ 95% confidence intervals. Asterisks and n.s. indicate significant (***) and non-significant (n.s.) differences.

significant (with $\alpha = 0.05$) differences between PA in mono- versus mixed culture, respectively.

Figure 2.

Temporal variation in pyoverdine levels for *P. aeruginosa* (PA), shown as difference between mixed cultures with *B. cenocepacia* (BC) and monocultures, in media with low (a), intermediate (b) and high (c) iron availability. Positive and negative peaks indicate higher pyoverdine levels in mixed and monocultures, respectively. Positive peaks only appeared under iron availability (a), suggesting that higher pyoverdine production might be a direct response to inter-specific iron competition. Negative peaks at later phases of competition appeared across all iron treatments, suggesting that they reflect more general responses to

competition. The amplitudes of both positive and negative peaks were fine-tuned in response to the level of inter-specific competition (illustrated by the purple bar). The level of competition was controlled by first manipulating the density of PA (expressed as percentage of $OD_{600} = 0.01$). For mixed cultures, we then added different amounts of BC (highest level of competition: 0.5 % PA vs. 99.5% BC; lowest level of competition 50 % PA vs. 50 % BC). In monocultures, we added respective amounts of NaCl buffer instead of BC. Pyoverdine fluorescence was measured over 48 hours and the mean differences of pyoverdine fluorescence of PA growing in mixed versus monoculture were calculated for each treatment and time point. The bold colored lines represent mean differences (7 to 8 replicates per treatment), whereas the faint colored areas show the 95% confidence intervals based on jackknifing (no overlap of the confidence intervals indicate significant differences between treatments).

Figure 3.

Comparison of growth parameters of *P. aeruginosa* (PA) in mixed cultures with *B. cenocepacia* (BC, blue circles) and in monocultures (red circles). Panels show the growth initial phase (IP, where growth rate = 0) (a-c), and maximal growth rate (d-f), measured in media with low (a, d), intermediate (b, e) and high (c, f) iron availability. Compared to monocultures, the IP was significantly shortened with high levels of competition (illustrated by the purple bar) under low iron availability (a), suggesting that an earlier onset of growth is a beneficial response to inter-specific iron competition. In contrast, the maximal growth

rate was significantly reduced in mixed cultures with high levels of competition across all iron treatments, suggesting that competition has a general negative effect for PA. The level of competition was controlled by first manipulating the density of PA (expressed as percentage of $OD_{600} = 0.01$). For mixed cultures, we then added different amounts of BC (highest level of competition: 0.5 % PA vs. 99.5% BC; lowest level of competition 50 % PA vs. 50 % BC). In monocultures, we added respective amounts of NaCl buffer instead of BC. Colored symbols represent single data points of 7 to 8 replicates per treatment, whereas black symbols show the mean $\pm$ 95% confidence interval. Colored lines depict the fits of the respective linear regressions.

Figure 4.

Temporal variation in single-cell *pvdA*-GFP gene expression in *P. aeruginosa* (PA) when growing either in mixed cultures with *B. cenocepacia* (BC) (blue circles) or in monocultures (red circles) under low iron availability. During the early growth phase (10 – 14 hours), the expression of *pvdA* (encoding an enzyme involved in pyoverdine synthesis via non-ribosomal peptide synthesis) was significantly upregulated in mixed compared to monocultures. At a later stage (20 hours), the *pvdA* expression levels switched, such that *pvdA* expression was significantly higher in mono- compared to mixed cultures. These results confirm that PA cells upregulate pyoverdine synthesis early on during inter-specific iron competition. Under iron-rich conditions (yellow circles), *pvdA* expression was low, demonstrating the functionality and sensitivity of our GFP-reporter. While faint colored circles show GFP fluorescence values for single cells ($n = 144$ to 642 per treatment and time point), bold circles and error bars depict means and 95% confidence intervals, respectively.

Colored lines depict the fits of the respective linear regressions. Initial PA density was 0.5 % (of OD600) for all experiments. In mixed cultures, we mixed 0.5 % with 99.5% BC, reflecting high levels of competition. Asterisks indicate significant (***) $P < 0.0001$ differences in *pvdA* expression between PA in mono- and mixed cultures in iron limited CAA medium.

Figure 5.

Comparison of the growth initiation phase (IP, a - c) and growth rate (e - g) of *P. aeruginosa* (PA) in mixed cultures with *B. cenocepacia* (BC, blue circles) and in monocultures (red circles), for strains that produce pyochelin, in addition to pyoverdine (wildtype), and strains that are deficient for pyochelin production (pyochelin knockouts). Since both species can produce the secondary siderophore pyochelin, in addition to their primary siderophore (pyoverdine for PA and ornibactin for BC), we checked whether pyochelin influences the fitness of PA in competition with BC. For IP, we recovered the same qualitative patterns for

the wildtype and the pyochelin-knockout strains, suggesting that pyochelin has no effect. Compared to monocultures, IP was significantly shortened with high levels of competition (0.5 % initial PA density) under low iron availability (a). This effect waned with low levels of competition (50 % initial PA density) and under intermediate (b) and high (c) iron availabilities. In contrast, the ability to produce pyochelin affected growth rate patterns under intermediate (e) and high (f) iron availabilities. Specifically, the reduced growth rate experienced by wildtype PA under high levels of competition disappeared in co-cultures of the pyochelin-knockout strains. The level of competition was controlled by first manipulating the density of PA (expressed as percentage of $OD_{600} = 0.01$). For mixed cultures, we then added different amounts of BC (highest level of competition: 0.5 % PA vs. 99.5% BC; lowest level of competition 50 % PA vs. 50 % BC). In monocultures, we added respective amounts of NaCl buffer instead of BC. Colored symbols represent single data points of 5 replicates per treatment, whereas black symbols show the mean $\pm$ 95% confidence interval.